The Enzymatic Synthesis of Nucleoside Analogues

By

Grace O'Connell

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Department of Chemistry

University of Warwick

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To My Parents

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DECLARATION

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously published. The work was carried out at the Department of Chemistry, University of Warwick and The Wellcome Research Laboratories, Beckenham, Kent, between October 1st 1990 and December 21st 1993 and has not been submitted for a degree at any other institution.

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I would like to thank Prof. David Crout, who stepped into the breach and supervised the rest of my PhD and writing up.

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ABSTRACT

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is a broad spectrum antiviral agent active against both DNA and RNA viruses. A range of 1,2,3-triazole, 1,2,4-triazole and benzotriazole analogues of ribavirin was synthesised using a crude extract of *N*-deoxyribosyltransferases from *Lactobacillus leichmannii* in the chemoenzymatic synthesis of nucleoside analogues with potential antiviral activity. *N*-Deoxyribosyltransferase catalyses the transfer of the 2-deoxyribose sugar between purine and pyrimidine bases. Of thirty two bases synthesised, twelve were found to act as acceptors in the *N*-deoxyribosyltransferase reaction. Nine 2'-deoxyribofuranosyl-1,2,4-triazole nucleoside analogues were isolated and characterised. Only one nucleoside showed any significant antiviral activity.

2'-Deoxyribavirin was found to possess antiviral activity against the influenza virus. Attempts were made to synthesise the nucleoside chemically from ribavirin on a larger scale, but without success. However, 5'-iodo-5'-deoxyribavirin and 5'-deoxyribavirin were synthesised, the former showed slight antiviral activity.

The specificities of the *N*-deoxyribosyltransferases for purine, pyrimidine and triazole bases are discussed, gathering together the findings of previous workers with the results presented in this thesis.

LIST OF ABBREVIATIONS

A	Adenine
ADP	Adenosine-5'-diphosphate
AIBN	Azobis(isobutyronitrile)
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture collection
ATP	Adenosine-5'-triphosphate
AZT	3'-Azido-3'-deoxythymidine
CI	Chemical ionisation
CMV	Cytomegalovirus
d	Doublet
dC	2'-Deoxycytidine
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
ddI	2',3'-Dideoxyinosine
DMAP	4-(Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EBV	Epstein Barr Virus
EI	Electron impact
EICAR	5-Ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide
FAB	Fast atom bombardment
FDA	Food and Drug Administration
FICAR	5-Fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide
g	Grams

GTP	Guanosine-5'-triphosphate
h	Hours
HIV	Human Immunodeficiency Virus
HPLC	High-pressure liquid chromatography
HSV	Herpes Simplex Virus
IMP	Inosine-5'-monophosphate
J	Coupling constant
lit.	Literature reference
m	Multiplet
m.p.	Melting point
mg	Milligrams
MHz	Megahertz
min	Minutes
ml	Millilitre
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSA	Mesitylenesulfonic acid
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance spectroscopy
nOe	Nuclear Overhauser enhancement
PDE	Phosphodiester
PIPES	[1,4-piperazinebis(ethanesulfonic acid)]
ppm	Parts per million
q	Quartet
RMP	Ribavirin-5'-monophosphate
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSV	Respiratory Syncytial Virus
RTP	Ribavirin-5'-triphosphate

s	Singlet
t	Triplet
TBAF	Tetrabutylammonium fluoride
TCA	1,2,4-Triazole-3-carboxamide
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TPDS	Tetraisopropyldisiloxane
UV	Ultra-violet
VZV	Varicella Zoster Virus
°C	Degrees Centigrade

Ac	Acetyl
Bn	Benzyl
Bu	Butyl
Bz	Benzoyl
Et	Ethyl
Me	Methyl
Ph	Aryl
Pr	Propyl
R	Alkyl

CHAPTER 1

INTRODUCTION

General Background

In developed countries up to sixty per cent of all illness is caused by viral infections such as influenza, herpes, hepatitis, rabies and mumps compared to only fifteen per cent resulting from bacterial infection.[1] Industry and government had been relatively inactive in preventing or alleviating the effects of viral infection by chemotherapy until acquired immune deficiency syndrome (AIDS)[2] brought unprecedented finance, manpower and determination to bear on solving problems associated with the human immunodeficiency virus (HIV), identified in the early 1980s.[3] Treatment of viral diseases is largely limited to prevention, i.e. vaccination, rather than cure, forestalling the infection by stimulating the immune system in advance rather than by attacking the virus directly. This is short-sighted considering the latency of herpes virus, infecting eighty per cent of the population, and the high antigenic variation of others such as the common cold.

Viruses were first identified in plant cells in the 1890s,[4] when it was shown that bacteria-free filtrates of tobacco plants infected with tobacco mosaic disease virus could produce the disease in healthy plants. In the same year the mammalian virus causing foot and mouth disease was identified.[5]

Viruses are packets of DNA or RNA contained in a protein coat. To replicate, the virus must first enter the cell by penetrating the cellular membrane, then commandeer the host cell's own genetic machinery to replicate itself, assemble the new virus particles and release them to

infect other cells. This intimate relationship with the biochemical mechanism of the host cell means that there are few unique functions for selective attack, that do not harm the host cell. In the replication of viral nucleic acids, there are certain enzymes which are specifically coded for by the virus. Most potential antiviral drugs (Table 1.1), the majority nucleoside analogues, target these enzymes and interrupt nucleic acid synthesis.

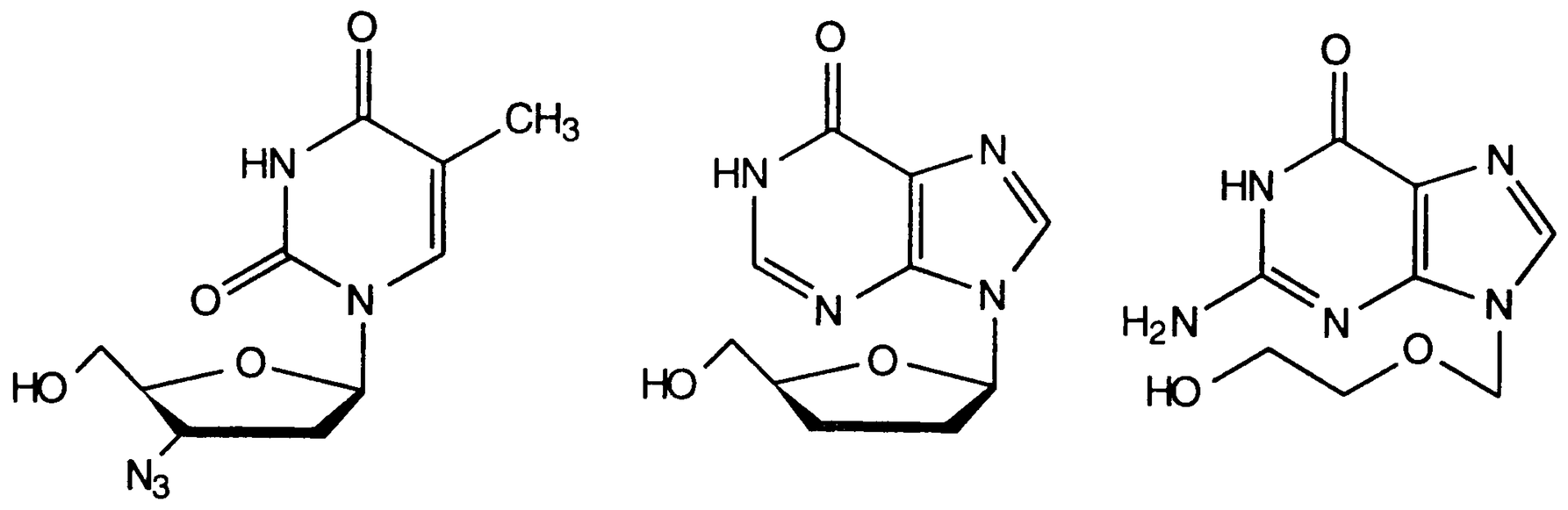
DRUG	**VIRUS**
Amantidine	Influenza A
Rimantidine	Influenza A
5-Iodo-2'-deoxyuridine	HSV
5-Trifluoromethyl-2'deoxyuridine	HSV
Adenine arabinoside	HSV
Acyclovir	HSV, VZV
Gancyclovir	Cytomegalovirus
5-Ethyl-2'-deoxyuridine	HSV (Germany)
5-Iodo-2'-deoxycytidine	HSV (France)
Ribavirin	Respiratory syncytial virus
3'-Azido-2',3'-dideoxythymidine	HIV-1
2',3'-dideoxyinosine	HIV-1
Penciclovir	HSV, VZV
Famciclovir	HSV, VZV

Table 1.1 Licensed antiviral drugs

Viruses and Antiviral Chemotherapy

Herpes viruses are double-stranded DNA viruses causing diseases such as

cold sores, chicken pox and genital sores. There are many nucleoside analogues targetted at this virus as it codes for many of the enzymes required in its own replication. The most successful is acyclovir[6] **3** (Fig. 1.1) which is now marketed by Wellcome as an over-the-counter drug.



1 AZT

2 ddI

3 Acyclovir

Fig. 1.1 The chemical structures of some antiviral nucleosides

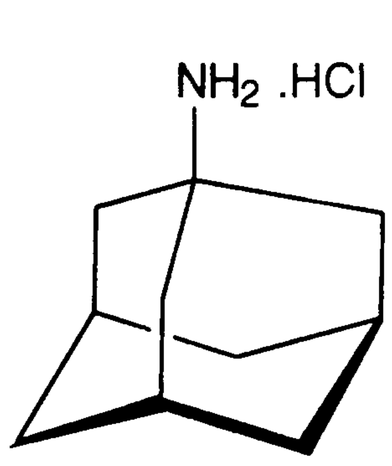
All the nucleoside analogues are phosphorylated to the triphosphate in the cell where they can act as chain terminators,[7-9] inhibit viral capping enzymes[10-12] or act as suicide substrates for some polymerases.[13]

The influenza viruses A, B and C of the family orthomyxoviridae provides a case where prevention by vaccination is not the complete answer. The protein coat of this enveloped RNA virus varies greatly due to point mutations or recombination of RNA between species making control by vaccine at best one step behind the virus.

Amantidine hydrochloride[14] **4** (Fig. 1.2), is a narrow spectrum agent, active only against influenza A virus. In 1966 it was the first antiviral drug approved by the FDA for the use against Asian influenza in the USA.

Rimantidine hydrochloride **5** (Fig. 1.2), an analogue with fewer side effects, is widely used in Russia[15, 16] and is shortly to be licensed in the

USA, for the treatment of influenza A virus.[17]



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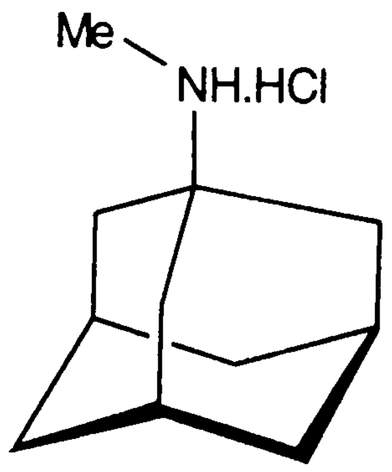
Amantidine hydrochloride



5

Rimantidine hydrochloride

Fig. 1.2 Drugs used in the treatment influenza

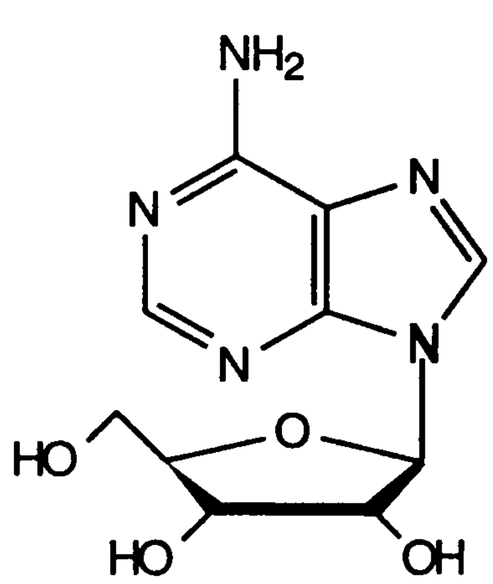
Both influenza drugs prevent infection by raising the pH of the endosomal/lysosomal compartments. The virus attaches to the cell as normal but once inside the secondary uncoating of the virus core is inhibited.[18, 19] This prevents RNA from reaching the nucleus where replication normally occurs.[20] Treatment works in seventy per cent of cases with influenza A, but neither drug is effective against influenza B. The potential neurotoxicity of amantidine hydrochloride and the rapid formation of resistant strains precludes their widespread use.

Ribavirin **7** is the next most effective inhibitor of influenza A and B replication and infection.[21]

Ribavirin

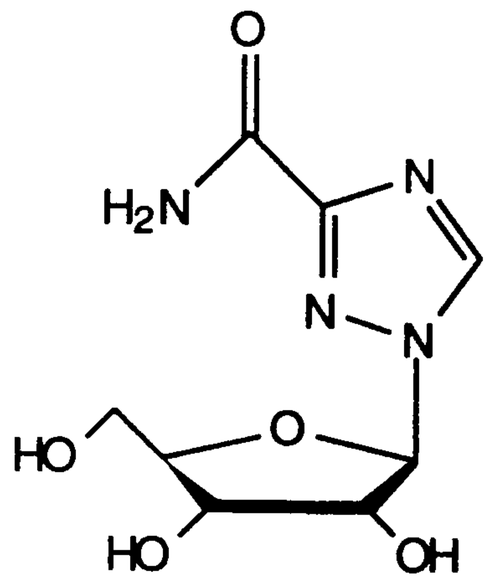
Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) **7** (Fig. 1.3), the first broad spectrum antiviral agent, was reported in 1972 as part of a program to prepare broad spectrum antiviral agents.[12, 22, 23] Since then, it has been studied in more animals and against more viruses than any other antiviral agent, but has the most pronounced activity against

orthomyxoviruses and paramyxoviruses.[24] It inhibits a wide range of DNA and RNA viruses *in vitro* and *in vivo*,[22, 25, 26] showing activity in cell culture against about eighty per cent of all viruses studied, with little cellular toxicity.[27] However Virazole™, marketed by ICN Pharmaceuticals, is only approved in the USA against severe respiratory syncytial virus (RSV) in young children.[28] It has shown teratogenicity in rats and hamsters,[29, 30] though not in baboons.[26, 31, 32]



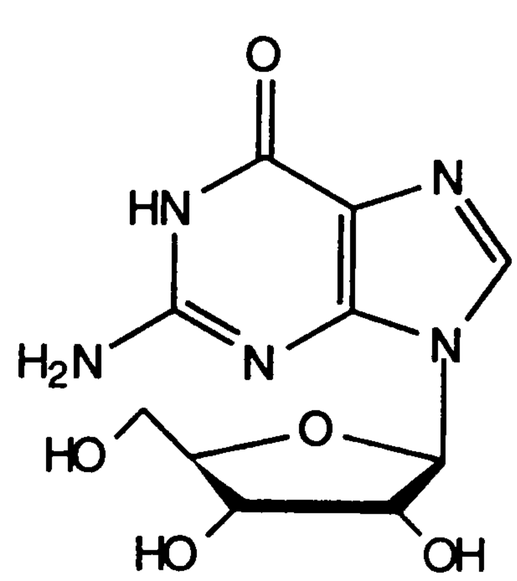
6

Adenosine



7

Ribavirin



8

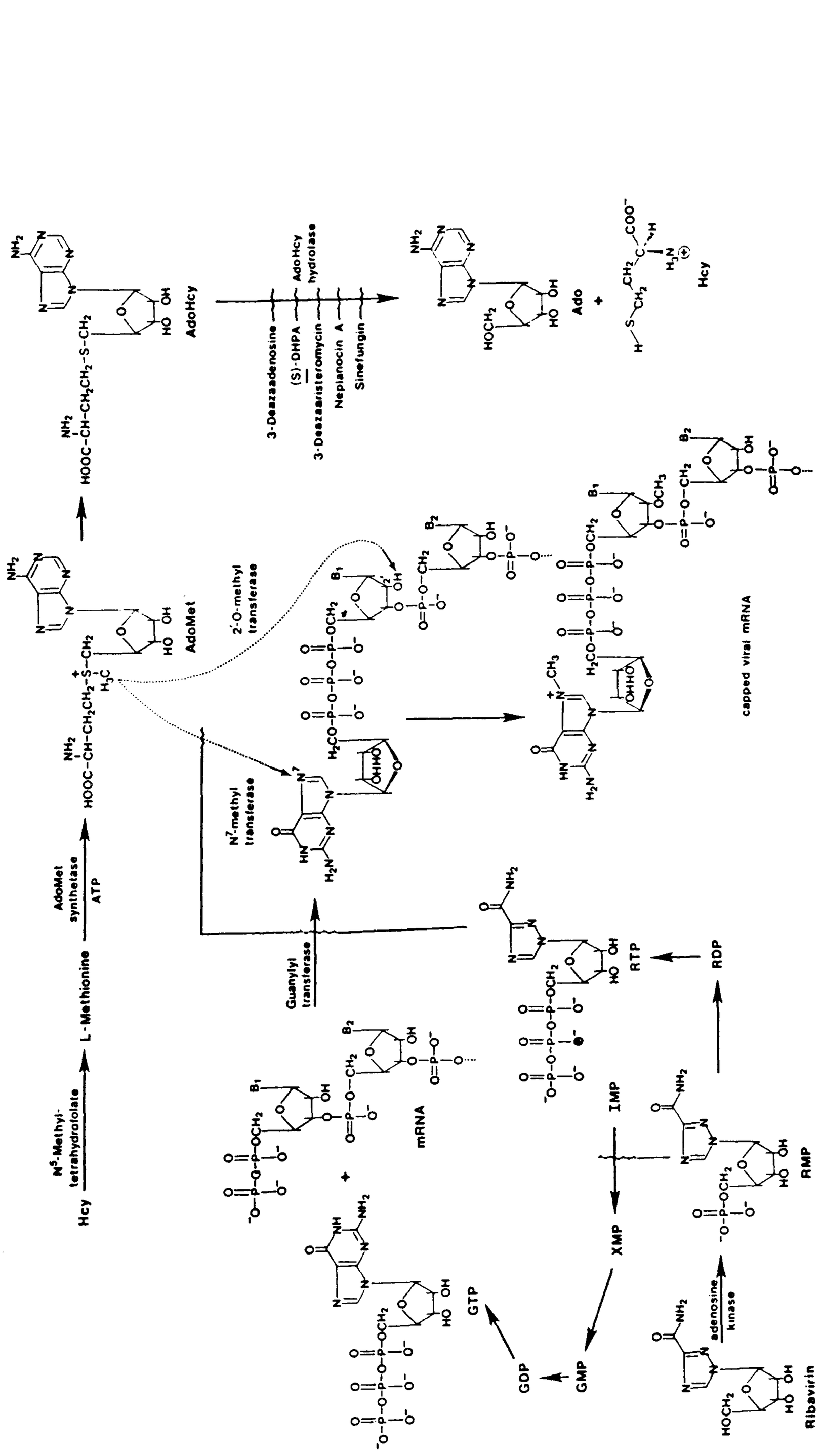
Guanosine

Fig. 1.3 Similarity of ribavirin to guanosine and adenosine

Ribavirin has a multipronged mode of attack (Scheme 1.1).[21] X-ray crystal studies show the striking similarity to guanosine **8** (Fig. 1.3), or on rotation about the amido group, to adenosine **6** (Fig. 1.3).[33] The inhibition of inosine monophosphate (IMP) dehydrogenase by ribavirin-5'-monophosphate (RMP), which has an affinity 70 times greater than IMP, results in a depletion of cellular GTP pools.[34] Ribavirin-5'-triphosphate (RTP), the most abundant form of ribavirin, can then more effectively competitively inhibit the viral specific RNA polymerase of some viruses by acting as a GTP or ATP substrate analogue.[35, 36] RTP inhibits the viral-specific mRNA capping enzymes, guanyltransferase and N^7-methyltransferase, interrupting viral protein synthesis[37] and inhibits

initiation and elongation of viral mRNA.[38] Resistance has only been detected recently in Sindbis virus mutates with an altered RNA guanyltransferase.[39] Ribavirin is not incorporated into the DNA or RNA of either mammalian or viral systems. Ribavirin is effective against viruses in plants, such as potato virus X, and in man, such as infectious hepatitis A[40] and potentiates the anti-HIV activity of dideoxynucleosides, e.g. dideoxyinosine.[41, 42]

The success of ribavirin has stimulated the chemical synthesis and antiviral testing of a large number of glycosides of related 5-membered heterocycles.[26, 43]



Scheme 1.1 Proposed mechanism of action of ribavirin[44]

Nucleoside Synthesis

Chemical Synthesis

There are three main strategies for the chemical synthesis of nucleoside analogues:[45, 46, 47]

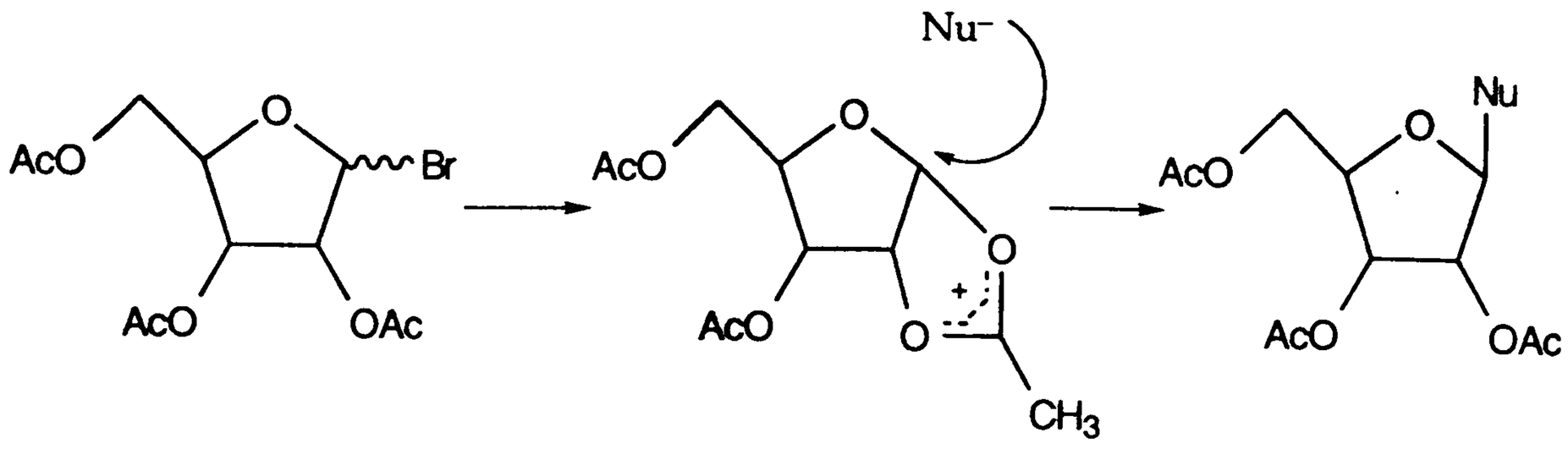
- the modification of a preformed nucleoside;
- the formation of the glycosidic bond by the fusion of a preformed base onto a sugar; or
- the *de novo* synthesis of a heterocyclic base onto a preformed nitrogen or carbon substituent at C-1 of the sugar moiety.

Formation of the Glycosidic Bond

Koenigs-Knorr Procedure

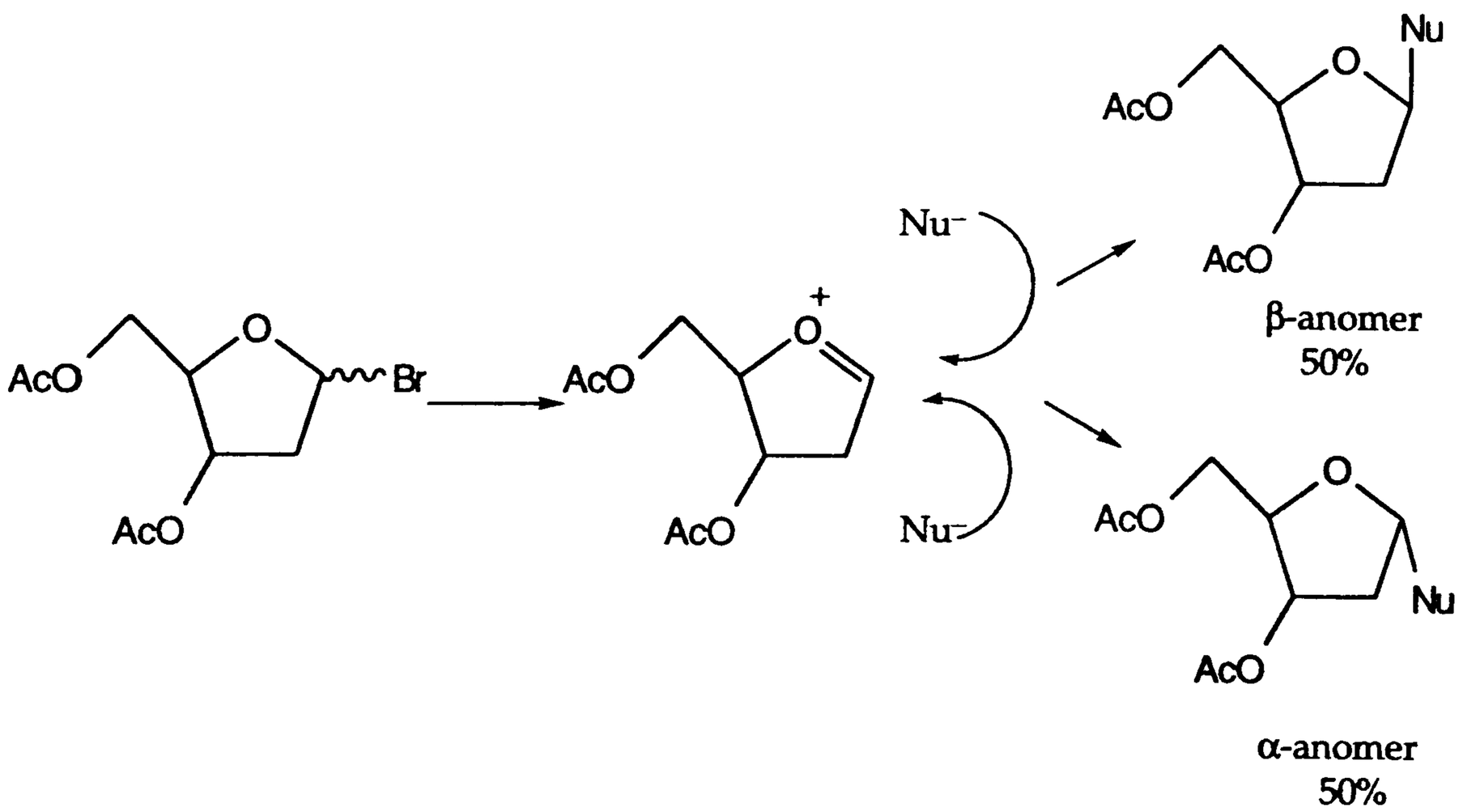
This reaction uses the presence of an acyloxy substituent at C-2 to produce selectively only the β-ribonucleosides. The initial sugar halide is formed from the reaction of the D-ribofuranose tetraacetate with hydrogen bromide in acetic acid to give 2,3,5-tri-*O*-acetyl-α-D-ribofuranosyl bromide.

Heavy metal salts of the bases catalyse the nucleophilic displacement of the halogen substituent from the C-1 of the protected sugar to give the 1,2 trans product, even though the reaction is unimolecular. Only the β-product is formed because the neighbouring trans-acetoxyl group at C-2 shields the α-face of the sugar (Scheme 1.2).



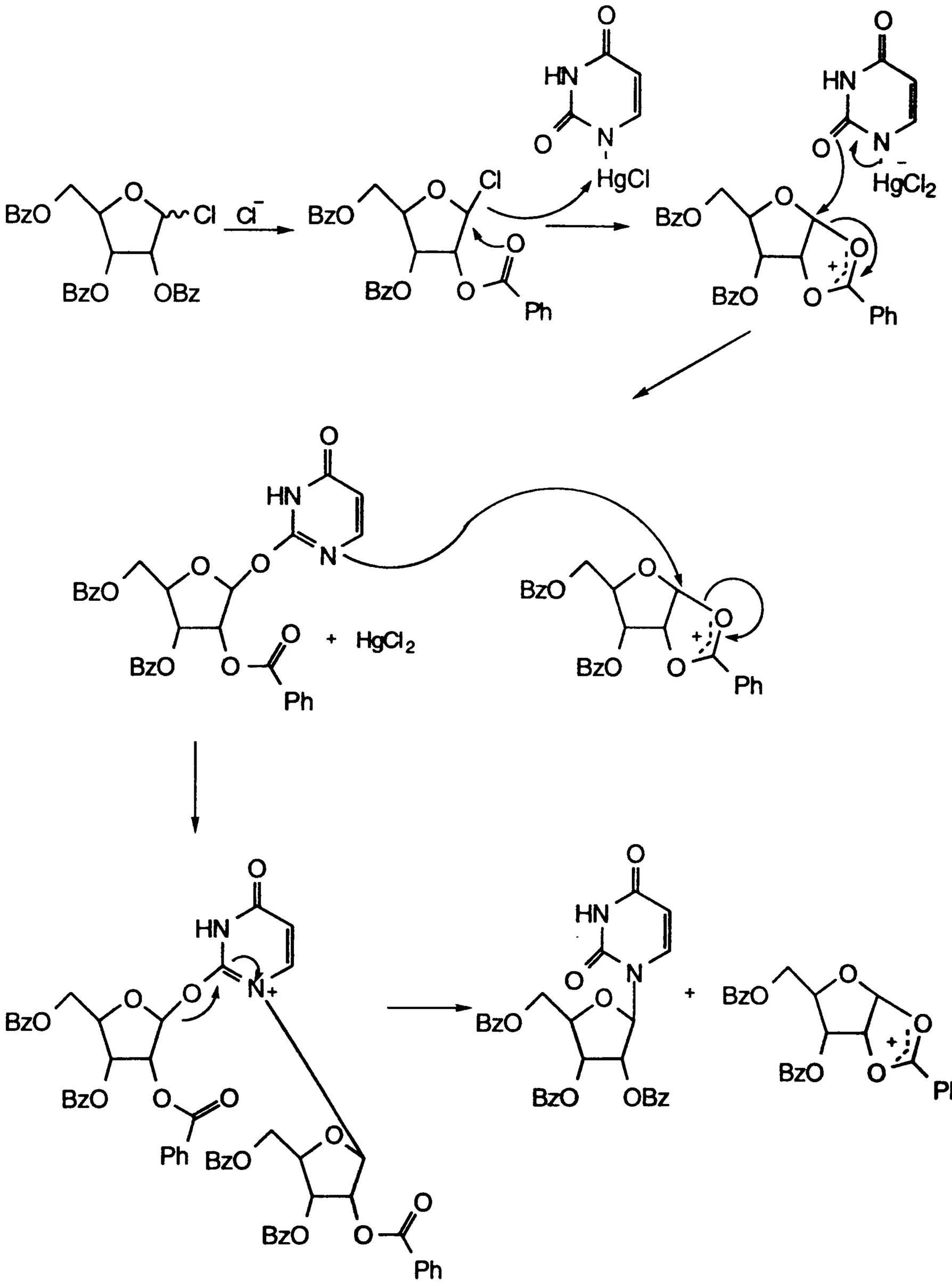
Scheme 1.2 Koenigs-Knorr displacement of halide

When the C-2 group is absent a mixture of α- and β-anomers results, confirming the stabilisation of the carbonium ion by neighbouring group participation (Scheme 1.3).



Scheme 1.3 Formation of both α- and β - anomers in the absence of a functional group at C-2

For the synthesis of 2'-deoxynucleosides and 2',3'-dideoxynucleosides other methods are, therefore, favoured.

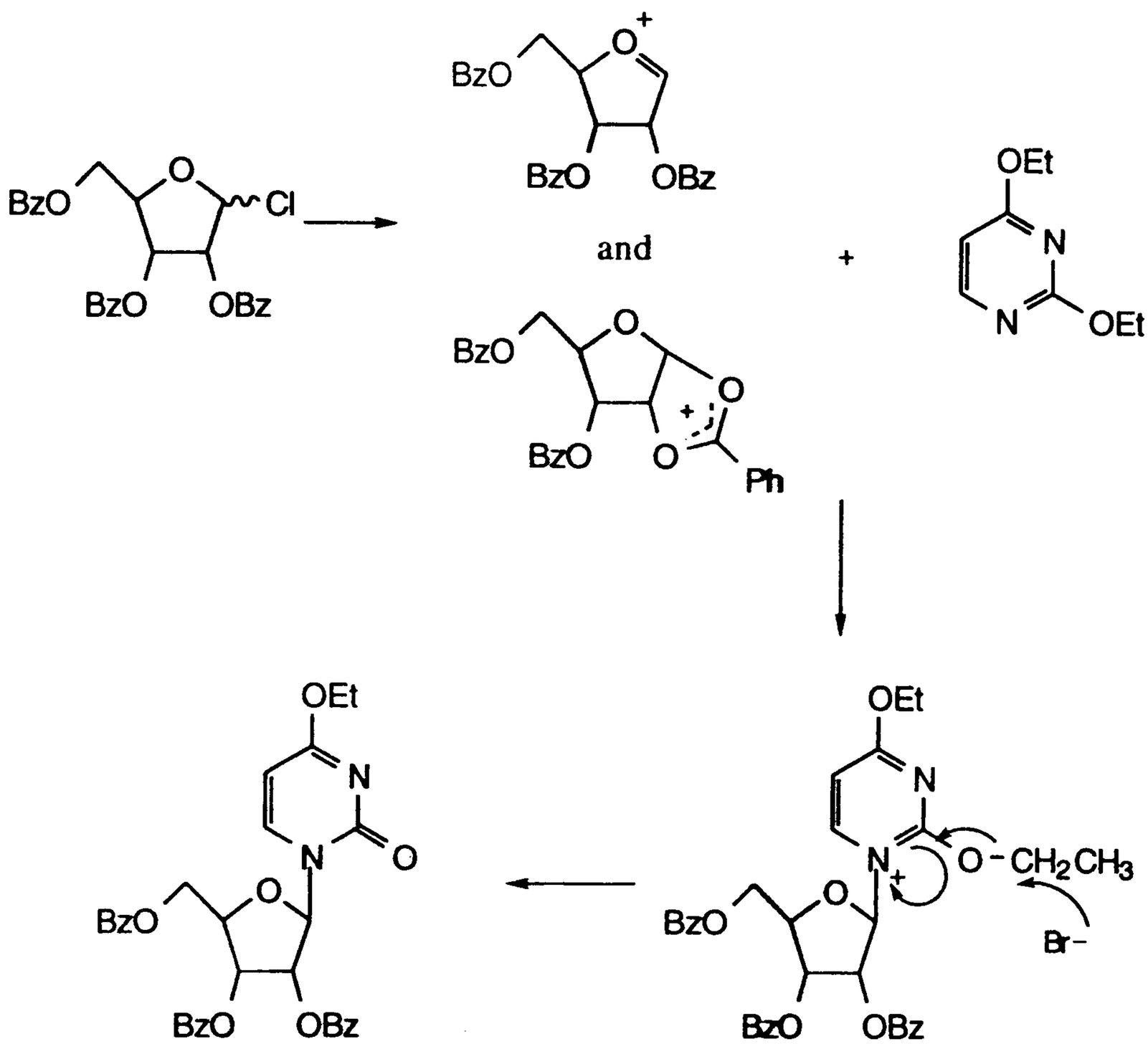


Scheme 1.4 Pyrimidine nucleoside synthesis

Hilbert-Johnson and Silyl Procedures

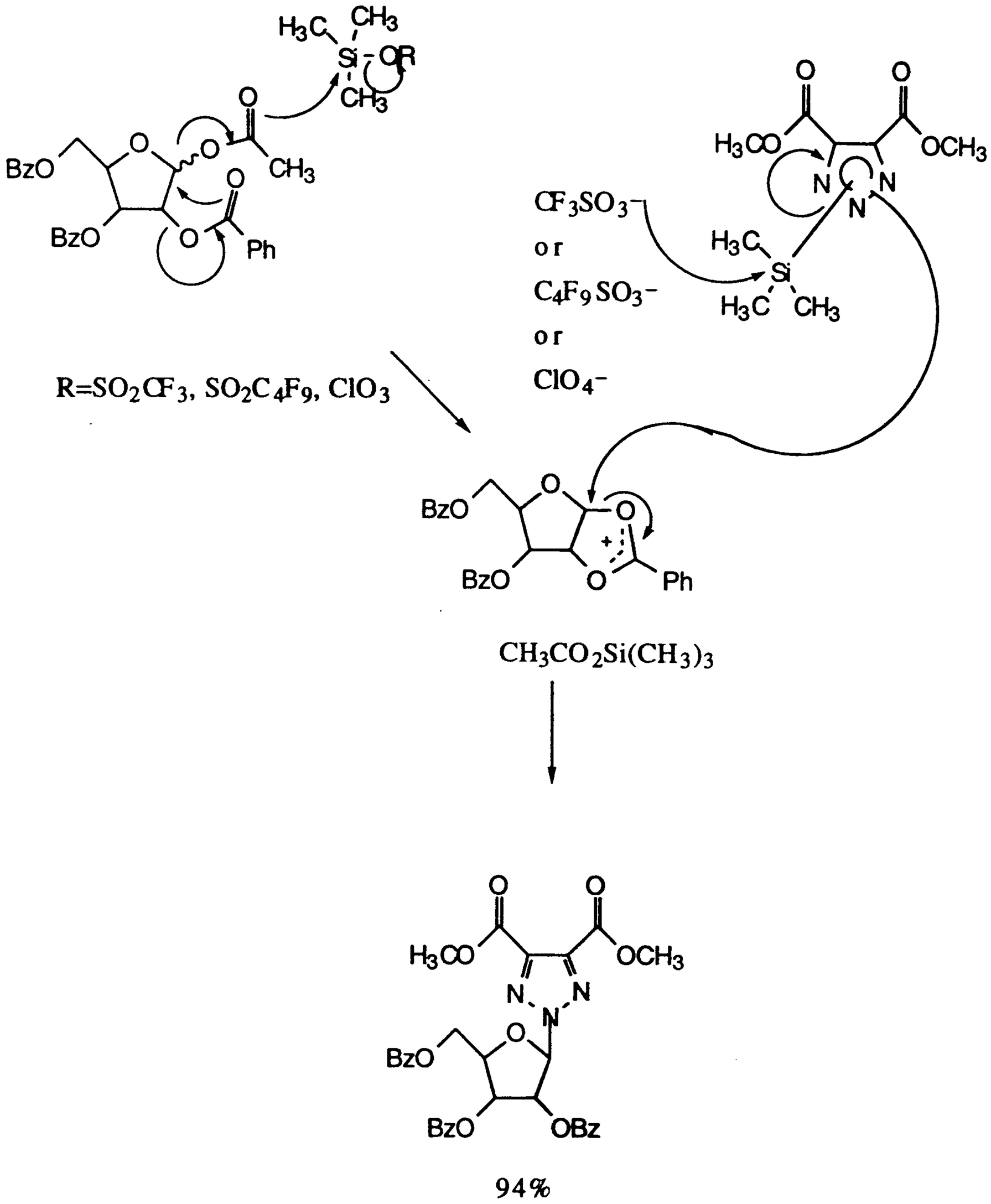
O-Substituted pyrimidines are sufficiently nucleophilic to react directly with halogeno-sugars without the need for electrophilic catalysis. In the

alkylation of a 2-alkoxypyrimidine with a halogeno-sugar the initial product is a quaternary salt which can then be used to give several nucleosides with different base moieties. Even with a ribose derivative an anomeric mixture is produced (Scheme 1.5).



Scheme 1.5 Hilbert-Johnson synthesis of nucleosides

This method was significantly improved by the introduction of silylated bases which are easily prepared, give homogeneous reaction mixtures and allow conversion of the intermediate products into a wide variety of nucleosides with modified bases. The early use of mercuric oxide gave way to other Lewis acid catalysts such as tin (IV) chloride and mercuric acetate, which were superseded by silyl esters of strong acids e.g. trimethylsilyl triflate (Scheme 1.6). These Friedel-Crafts-catalysed reactions are fast and high yielding.[48-50]

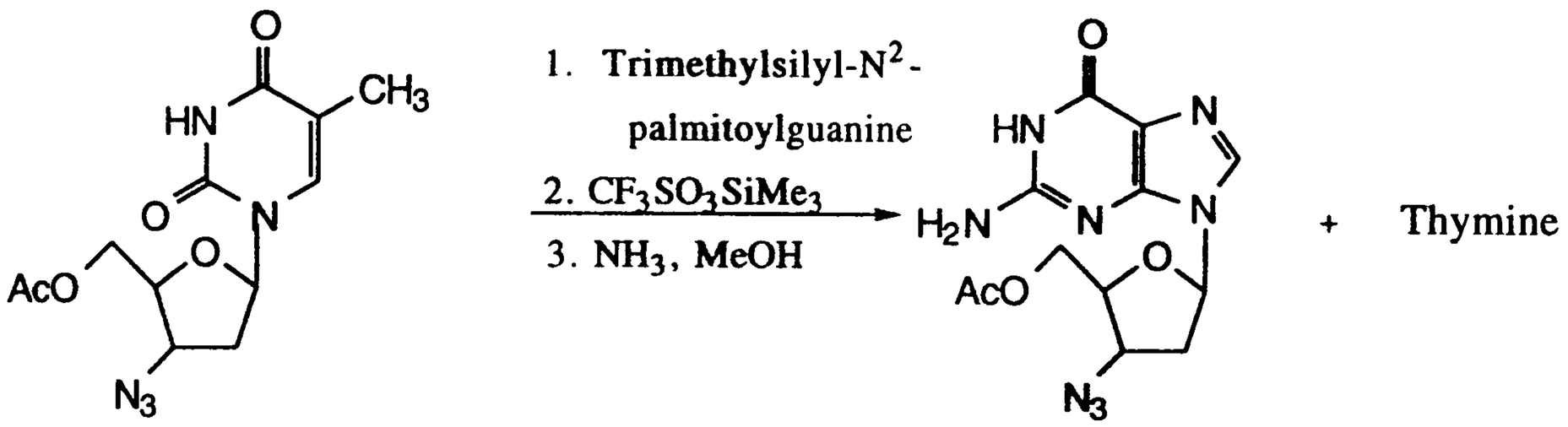


Scheme 1.6 Friedel-Crafts-catalysed nucleoside synthesis[51]

With ribose sugars the β-nucleoside is formed exclusively. In the synthesis of 2'-deoxynucleosides a nonpolar solvent will promote the S_N2 mechanism giving predominantly the β-nucleosides.[52] Many nucleoside analogues have been made in this way,[53] but the lack of regioselectivity and stereoselectivity and the need for protecting groups can reduce yields.

Transglycosylation

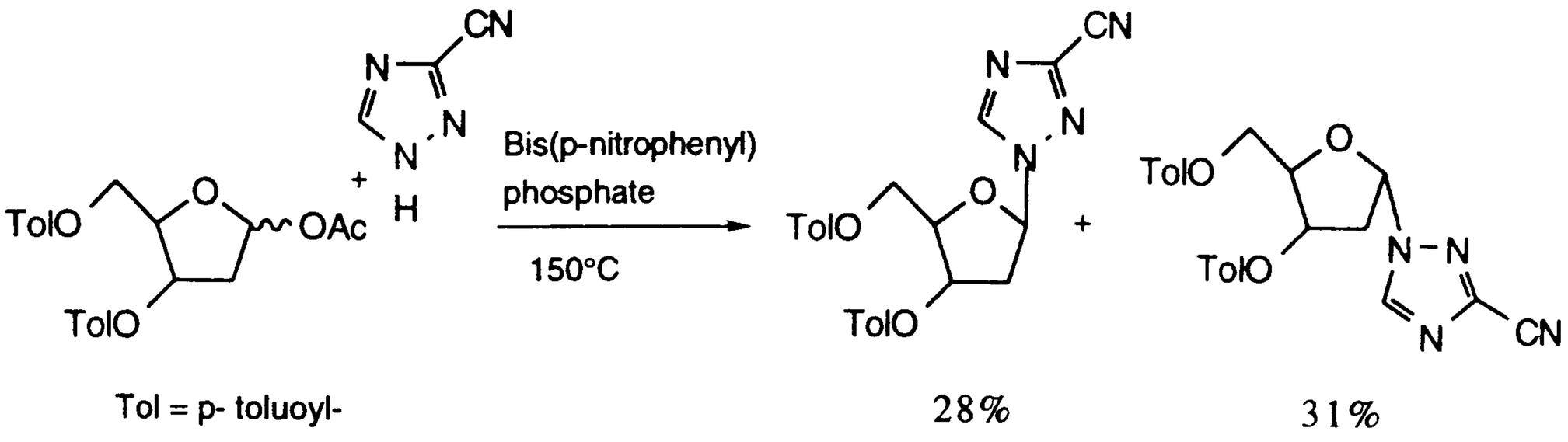
Transglycosylation is the transfer of a sugar residue from one base to another. It is most effective for transferring sugars from pyrimidines (which are π-deficient heterocycles) to the more basic purines (π-excessive heterocycles), by an S_N1 reaction mechanism (Scheme 1.7).[54]



Scheme 1.7 Synthesis of nucleosides by transglycosylation

Fusion

A melt of the 1-acetoxy sugar and a suitable base *in vacuo*, with a trace of acid catalyst gives the nucleoside in acceptable yields. This method is best for weakly basic heterocycles having a low melting point and has been used in many convergent syntheses of triazole nucleoside analogues (Scheme 1.8).[55-57]



Tol = p- toluoyl-

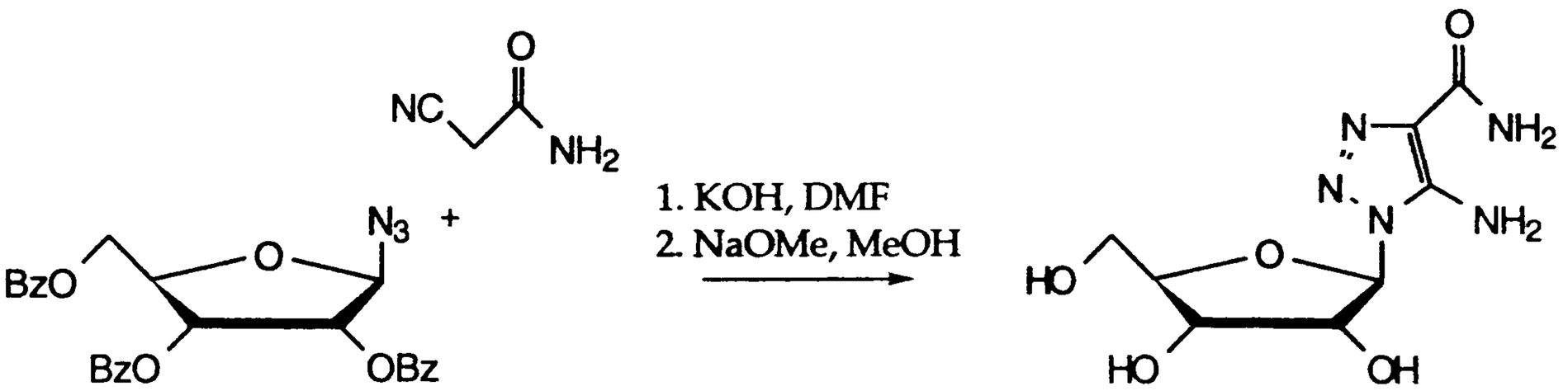
28%

31%

Scheme 1.8 Fusion synthesis of nucleosides[58]

Construction of the Base onto the Sugar at C-1

The advantages of building the base on to the sugar moiety are that the position of attachment of the glycosyl residue is unambiguous and the β-configuration at C-1 will usually be conserved in the product nucleoside. However, anomerisation of glycosyl azides has been reported.[59] This route is often employed for the synthesis of *C*-nucleosides and carbocyclic nucleosides which can therefore be derived from starting materials other than sugars or other carbohydrates (Scheme 1.9).[45, 60]



Scheme 1.9 Synthesis of a nucleoside by building the base onto the sugar

Modification of Nucleosides

Hundreds of reactions have been used to transform the base of a preformed nucleoside.[46, 61, 62, 63] Unfortunately modification of the sugar moiety often necessitates multistep procedures involving protection

sugar moiety often necessitates multistep procedures involving protection and deprotection to control stereoselectivity and regioselectivity.[47, 64]

Enzymatic Synthesis

Many nucleoside analogues are difficult to synthesise chemically because of the need for both modified base and sugar residues. The multistage processes are often difficult and time consuming giving mixtures of products in poor yields. The advantages of enzymatic methods over purely chemical methods are their efficiency, selectivity and the mildness with which they catalyse reactions. The broad substrate specificities of some enzymes allow reactions to be performed on analogues of natural substrates in both aqueous and non-aqueous solvents. Enzymes can be used to replace existing chemical processes or in combination with chemical steps to improve existing methods. Difficulties may arise in scale-up production due to the concentration of substrates or of the presence of other species in the reaction solution. Immobilisation on a support material can stabilise the enzyme and a small quantity of enzyme can be used repeatedly to synthesise substantial amounts of product and, it is recoverable at the end. A large range of products can be prepared under these mild conditions in good yields making it a convenient method for the small scale synthesis of nucleoside analogues in screening programmes.[65-69]

Enzymatic Synthesis of Nucleoside Analogues

Enzymatic Modification of Sugar or Base Residues

Enzymes can be used to modify existing nucleosides by selective protection

and deprotection of hydroxyl groups.[70-73] By far the most useful applications have been the resolution of racemic nucleoside analogues by sugar modification[74] and base modification using adenosine deaminase.[75, 76]

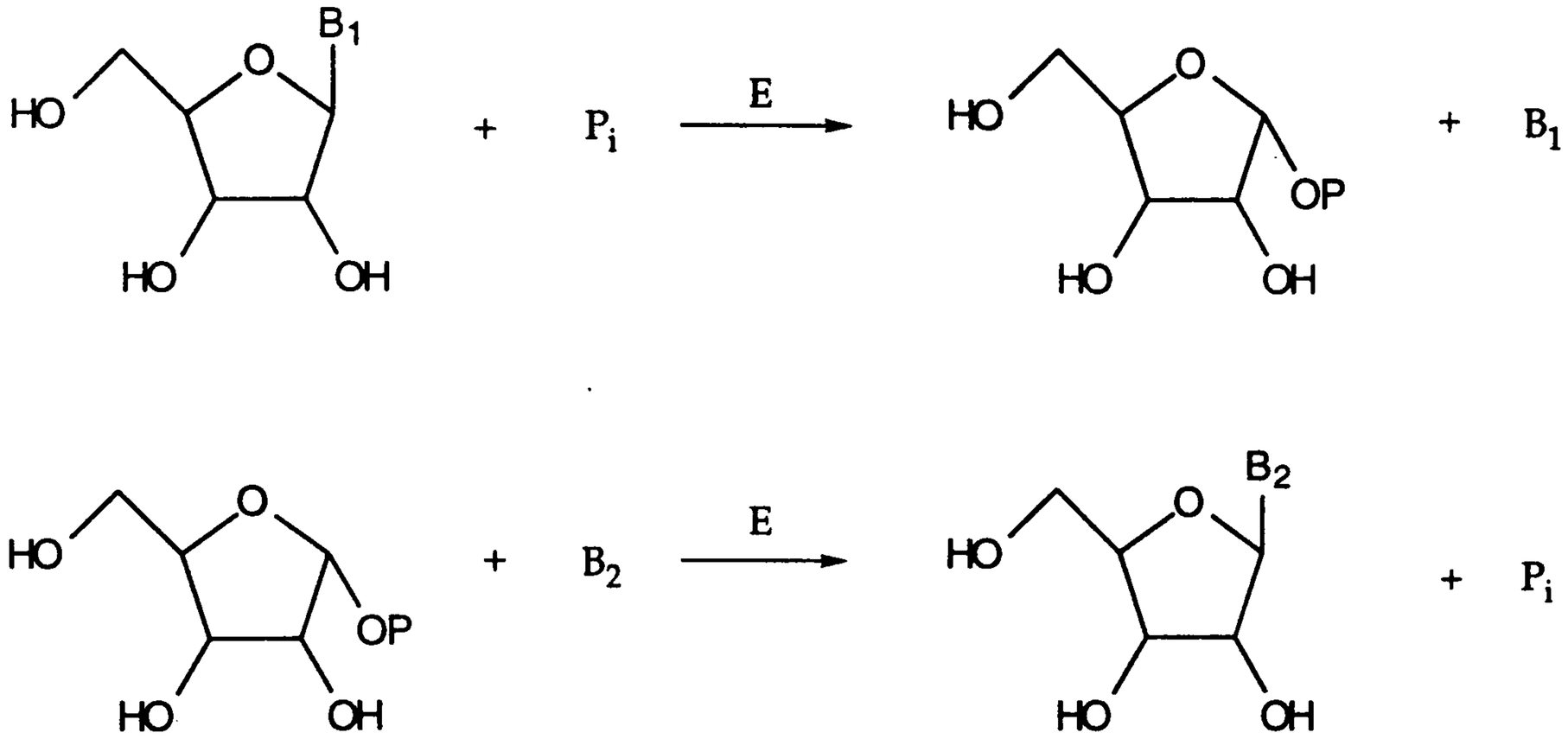
Glycosyl Transfer Reactions

Two main classes of enzyme have been used to synthesise nucleoside analogues by transferring glycosyl residues from a donor nucleoside to an acceptor base: nucleoside phosphorylases and *N*-deoxyribosyltransferases.[69]

Nucleoside Phosphorylases

Nucleoside phosphorylases catalyse the reversible phosphorolysis of ribonucleosides or deoxyribonucleosides affording ribose-1-phosphate or deoxyribose-1-phosphate and a purine or pyrimidine base. Addition of an acceptor base can lead to the formation of a new nucleoside with the equilibrium lying well in favour of nucleoside formation. Since the discovery of nucleoside phosphorylase and a general description of the mechanism[77] this method of nucleoside synthesis has been widely used. Both pyrimidine and purine nucleoside phosphorylases are known. They can be obtained from mammalian and bacterial sources and their substrate specificities can vary with the source.[78]

Two strategies have generally been used (with ribose as the sugar moiety in the example of Scheme 1.10). The first involves isolation of the ribose-1-phosphate from a nucleoside donor and a high concentration of phosphate. This intermediate is then used as the glycosyl donor with respect to an added pyrimidine or purine base.



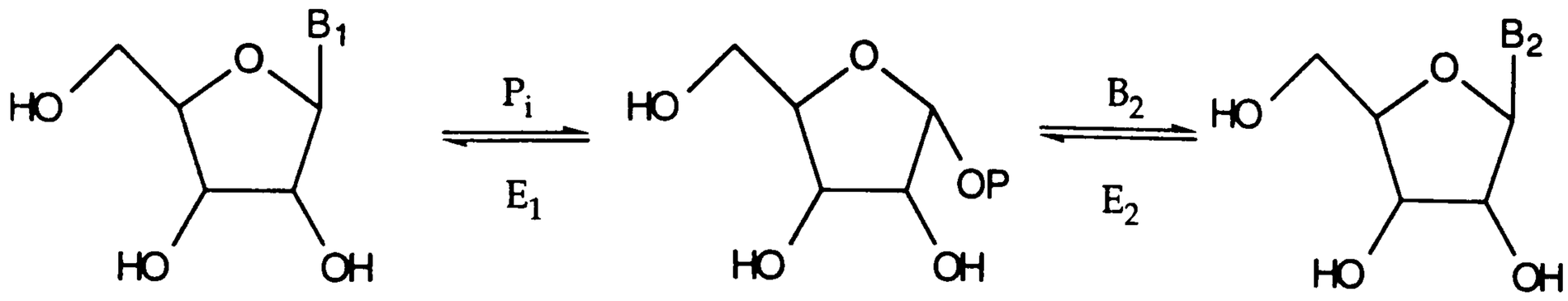
B_1 = purine (or pyrimidine) base

B_2 = purine (or pyrimidine) base

E = nucleoside phosphorylase

Scheme 1.10 Two-stage synthesis of nucloesides using nucleoside phosphorylases

The second strategy involves a one-pot exchange of one base for another in the presence of a catalytic amount of inorganic phosphate without the isolation of the intermediate ribose-1-phosphate (Scheme 1.11).



B_1 = pyrimidine

B_2 = purine

E_1 = pyrimidine nucleoside phosphorylase

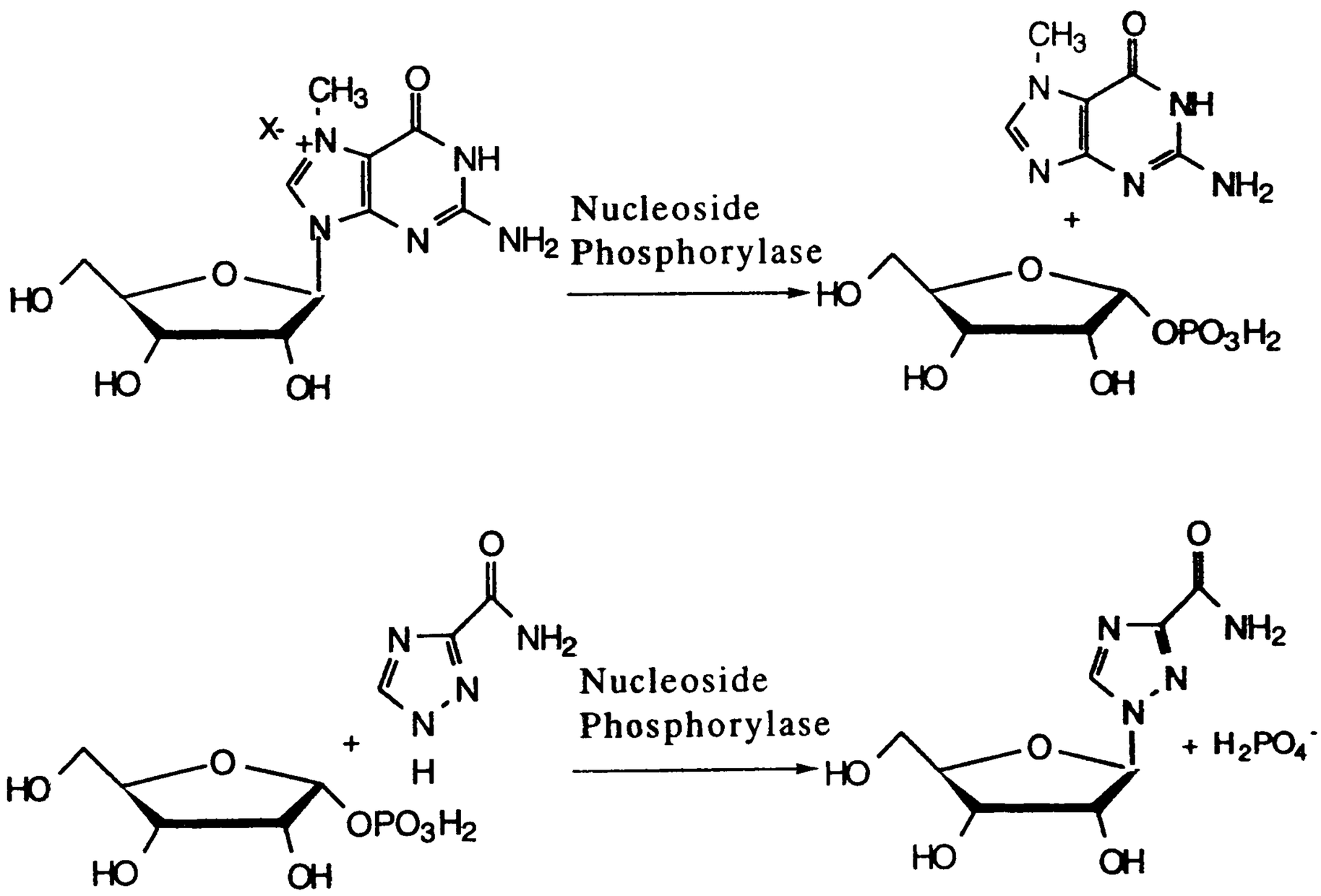
E_2 = purine nucleoside phosphorylase

Scheme 1.11 One-pot synthesis of nucleosides using nucleoside phosphorylases

Both methods have been used in the small scale production of a variety of nucleoside analogues.[69] The first strategy is the most general. The second[79] has limitations. For example, an equilibrium mixture of starting and product nucleosides can be formed from which the desired nucleoside must be separated. In other cases, the base released by the glycosyl donor may act as a competitive inhibitor of the enzyme and prevent the base analogue from binding. The problem of inhibition can be avoided by using a pyrimidine nucleoside as the glycosyl donor and a purine base as the acceptor, but both a pyrimidine and a purine nucleoside phosphorylase are then required.

Both methods have disadvantages as they either require the presence of both pyrimidine and purine nucleoside phosphorylases or the isolation of the intermediate ribose-1-phosphate. These problems can be overcome and the yields of the coupled enzymatic reaction can be greatly increased by the use of an irreversible donor nucleoside.[80] By using the salt of 7-methylguanosine, as the glycosyl donor, the first stage of the

phosphorolysis reaction is made irreversible. The cleavage of the glycosyl bond produces a neutral base molecule which lacks a tautomeric proton on N-7. The formation of the positively charged nucleoside in the reverse direction would be highly unfavourable. Therefore, the 7-methylguanine does not function as an acceptor base in the reverse reaction and so the intermediate ribose-1-phosphate formed is available exclusively for the formation of product nucleoside. Only one of the nucleoside phosphorylases, not both, is required and as the 7-methylguanine is very insoluble, purification of the product nucleoside is very easy. The effectiveness of this approach was demonstrated in the synthesis of ribavirin using 1,2,4-triazole-3-carboxamide (TCA) as the acceptor base (Scheme 1.12).

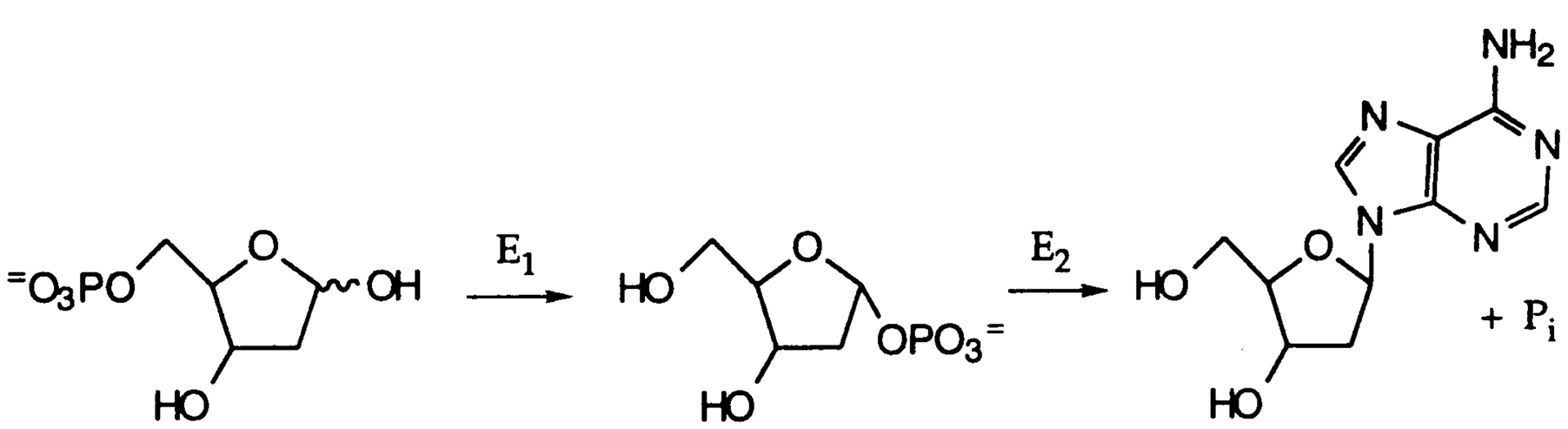


Scheme 1.12 Use of 7-methylguanosine as an irreversible donor in the synthesis of nucleoside analogues[80]

The nucleoside phosphorylases have been found to accept a wide range of

nucleoside and base analogues as substrates. The enzymes are able to tolerate a high degree of modification in the base component but only a limited amount of modification of the sugar moiety. So far nucleoside analogues have been synthesised with D-arabinofuranosyl, 2-aminoribose, 2-deoxy-3-aminoribose, and 5-deoxyribose as the sugar moiety. The nucleoside analogues which have been made by nucleoside phosphorylases have been reviewed.[69]

Recently, a new route to the enzymatic synthesis of nucleoside analogues has been described which involves the coupling of nucleoside phosphorylase with phosphopentomutase (Scheme 1.13).[81] The advantage of this method is that the pentose-5-phosphate is easier to prepare and more stable than the corresponding ribose-1-phosphate. This method is limited by the mutase which only accepts 2-deoxyribose-5-phosphate (the natural substrate), D-ribose and D-arabinose-5-phosphate.



E_1 = Phosphopentomutase

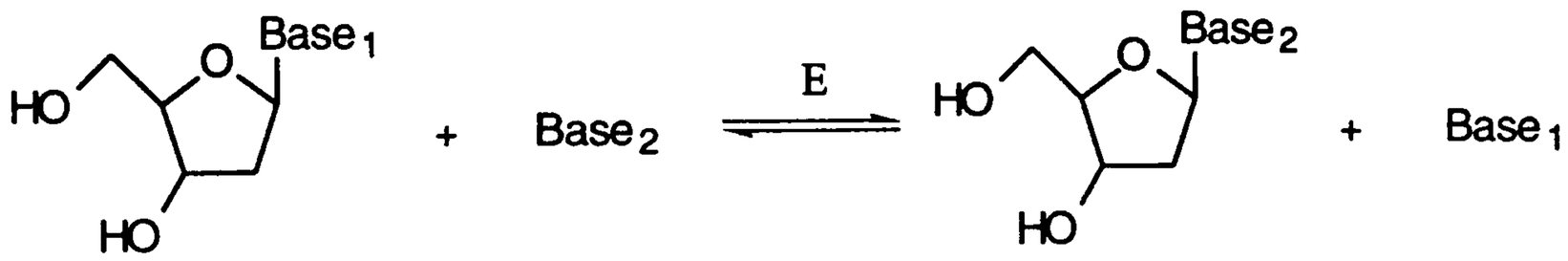
E_2 = Nucleoside phosphorylase and adenine

Scheme 1.13 Synthesis of nucleosides using nucleoside phosphorylase and phosphopentomutase

N-Deoxyribosyltransferases

The other class of enzymes used in nucleoside synthesis are the *N*-

deoxyribosyltransferases which were first reported by MacNutt.[82] These enzymes were shown to catalyse deoxyribosyl transfer from 2'-deoxyribonucleosides to free purines and pyrimidines without the need for phosphate ions. The reaction involves the direct transfer of the 2-deoxyribose group from one heterocyclic base to another without the formation of a 2-deoxyribose-1-phosphate intermediate (Scheme 1.14). The use of 2-deoxyribose-1-phosphate as the glycosyl donor did not result in the formation of any 2-deoxyribonucleosides. Further experiments were performed to show that the reaction mechanism did not involve hydrolysis of the glycosidic bond followed by resynthesis with the donor heterocyclic base to give the new nucleoside[82], nor the transamination of 2'-deoxyinosine to produce 2'-deoxyadenosine.[83] The reaction catalysed was in fact trans-*N*-glycosylation.



$Base_1$/ $Base_2$ = purine or pyrimidine

E = *N*-deoxyribosyltransferase

Scheme 1.14 Reaction of *N*-deoxyribosyltransferase

An extensive screening programme has been undertaken to investigate the occurrence and specificity of this enzyme.[84] Nucleoside deoxyribosyltransferases are present in appreciable levels in some organisms, notably the family lactobacillacae, which do not contain high levels of the nucleoside phosphorylase enzymes and require *N*-deoxyribosyltransferases to produce 2-deoxynucleosides for growth. This activity has been found in *Lactobacillus helveticus*,[85] *L. delbruecki*,[86] *L.*

acidophilius[87] and *L. lactis*,[88] but is not present in extracts of *L. casei*,[88] other microbes or animals.[89] However, recently a purine-2'-deoxyribosyltransferase activity has been purified from a protozoa, *Crithidia luciliae*.[90]

Many procedures for the purification or partial purification of the *N*-deoxyribosyltransferases from various lactobacilli have been reported.[86, 87-89, 91, 92] The presence of two distinct enzymes in *L. helveticus* was first shown by Beck.[88] These were isolated using affinity chromatography with two types of ligand.[92, 93] *N*-Deoxyribosyltransferase I catalysed the transfer of 2-deoxyribose residues exclusively between purine bases, whereas *N*-deoxyribosyltransferase II catalysed the transfer of 2-deoxyribose residues between purine and pyrimidine bases (Fig. 1.4).

N-Deoxyribosyltransferase I

dRib-Pur(1) + Pur(2) ⇌ dRib-Pur(2) + Pur(1)

N-Deoxyribosyltransferase II

dRib-Pur(1) + Pur(2) ⇌ dRib-Pur(2) + Pur(1)

dRib-Pur + Pyr ⇌ dRib-Pyr + Pur

dRib-Pyr(1) + Pyr(2) ⇌ dRib-Pyr(2) + Pyr(1)

dRib = 2-deoxy-D-ribose

Pur = Purine

Pyr = Pyrimidine

Fig. 1.4 Activities shown by the two transferase enzymes

Detailed kinetic studies of both transferase enzymes from *L. helveticus* were performed and the initial velocity studies showed that the 2-

deoxyribosyl transfer reactions both proceed *via* a ping-pong bi-bi mechanism (Fig. 1.5).[94] The kinetic mechanism is ping-pong because the first product is released before the second substrate combines with the enzyme and it is bi-bi because the reaction is bimolecular.[95, 96]

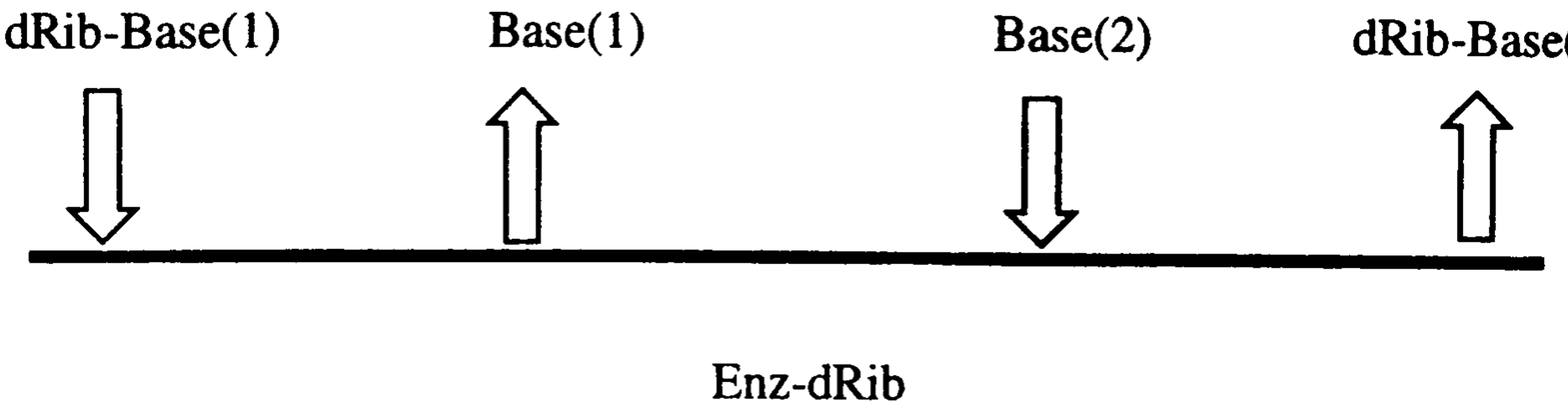


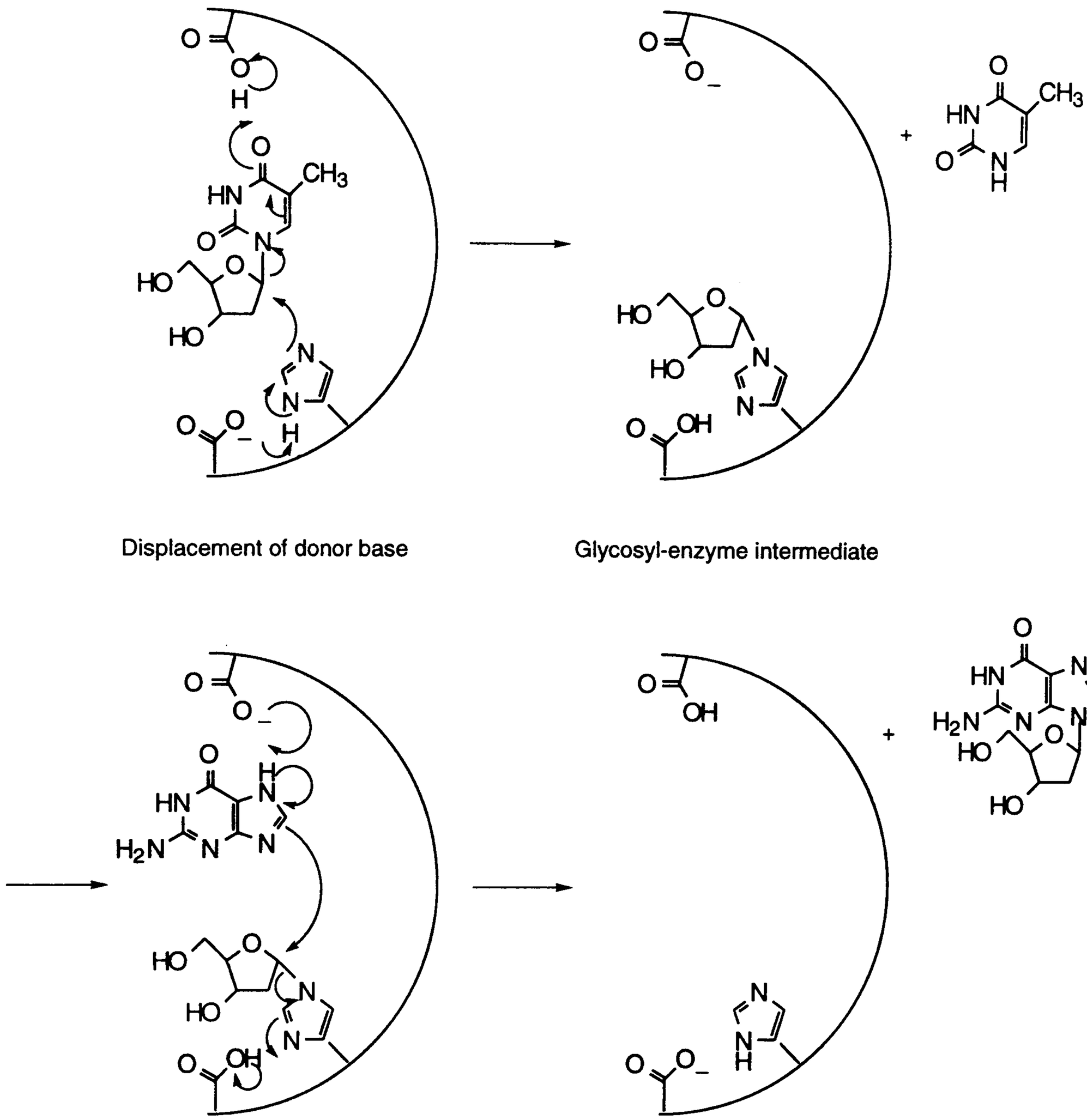
Fig. 1.5 Ping-pong bi-bi mechanism of *N*-deoxyribosyltransferase

The donor nucleoside combines with the enzyme and the glycosyl bond is cleaved. The base is released, leaving a glycosyl-enzyme intermediate. The acceptor base enters the active site and combines with the glycosyl-enzyme intermediate. Finally, the new nucleoside is released leaving the free enzyme. This mechanism assumes the existence of a stable glycosyl-enzyme intermediate in which the 2-deoxyribosyl moiety undergoes a double inversion.

A detailed study of the substrate specificities of the *N*-deoxyribosyl-transferases from *L. helveticus* has been described.[97] A wide range of heterocyclic bases were accepted by the enzyme but only a very limited number of nucleosides with modified sugar residues were accepted as donors.

Two distinct *N*-deoxyribosyltransferase activities were purified from *Lactobacillus leichmannii* and their substrate specificities were investigated.[98] As with *Lactobacillus helveticus*, these enzymes were

capable of accepting a wide number of heterocyclic bases as substrates but showed little tolerance towards donor nucleosides with modified sugar residues. The transfer reaction proceeded *via* a ping-pong bi-bi mechanism and kinetic and radiolabelling experiments provided preliminary support for the existence of a covalent glycosyl intermediate. Chemical modification of *N*-deoxyribosyltransferase II with specific chemical reagents suggested that histidine and/or carboxyl residues may participate in binding and catalysis at the active site of the enzyme. A proposed mechanism was suggested that is consistent with the information ascertained so far and highlights the double-inversion of the 2-deoxyribosyl moiety to produce β-nucleosides exclusively (Scheme 1.15).[98]



Scheme 1.15 Proposed mechanism of *N*-deoxyribosyltransferase[98]

N-Deoxyribosyltransferase has been purified and characterised from *Leuconostoc mesenteroides* subspecies *cremoris*.[99] A single multifunctional enzyme capable of carrying out the transfer of the 2-deoxyribosyl moiety from either pyrimidine or purine nucleosides to either pyrimidine or purine bases was isolated. Kinetic studies carried out on the purified enzyme showed that the transfer reaction proceeded *via* a ping-pong bi-bi mechanism. Further evidence for the existence of a

glycosyl intermediate was provided by radiolabelling studies.

Microcrystals of nucleoside deoxyribosyltransferase obtained from *Lactobacillus helveticus* have been reported,[85] but these were not of sufficient quality to be used for X-ray studies. Crystals of recombinant *N*-deoxyribosyltransferase from *Lactobacillus leichmannii* have been grown from ammonium sulfate,[100] though the type of reaction it catalysed was not specified. Molecular weight studies revealed a molecular weight for the intact enzyme of about 110,000 and a subunit molecular weight of about 18,000. Thus, the enzyme is a hexamer of six identical subunits.

Function of *N*-Deoxyribosyltransferase Activity

Within cells, nucleosides can be synthesised either: by the *de novo* synthesis or by the salvage pathway. In the *de novo* pathway the purine and pyrimidine bases are constructed onto the ribose moiety in several steps. This, overall, is a high energy process. A much more efficient method is the salvaging of bases produced by the hydrolysis of nucleic acids and nucleotides. The metabolic function of the *N*-deoxyribosyltransferase is believed to be part of nucleic acid synthesis.[101] The effects of varying vitamin B_{12}, 2'-deoxyribonucleoside or free purine and pyrimidine bases have been investigated. It was shown that vitamin B_{12} participates in the reduction of ribonucleosides to 2'-deoxyribonucleosides.[102-106] The *N*-deoxyribosyltransferase activity serves as an alternative pathway for 2'-deoxynucleoside synthesis in the absence of vitamin B_{12} (Fig. 1.6).

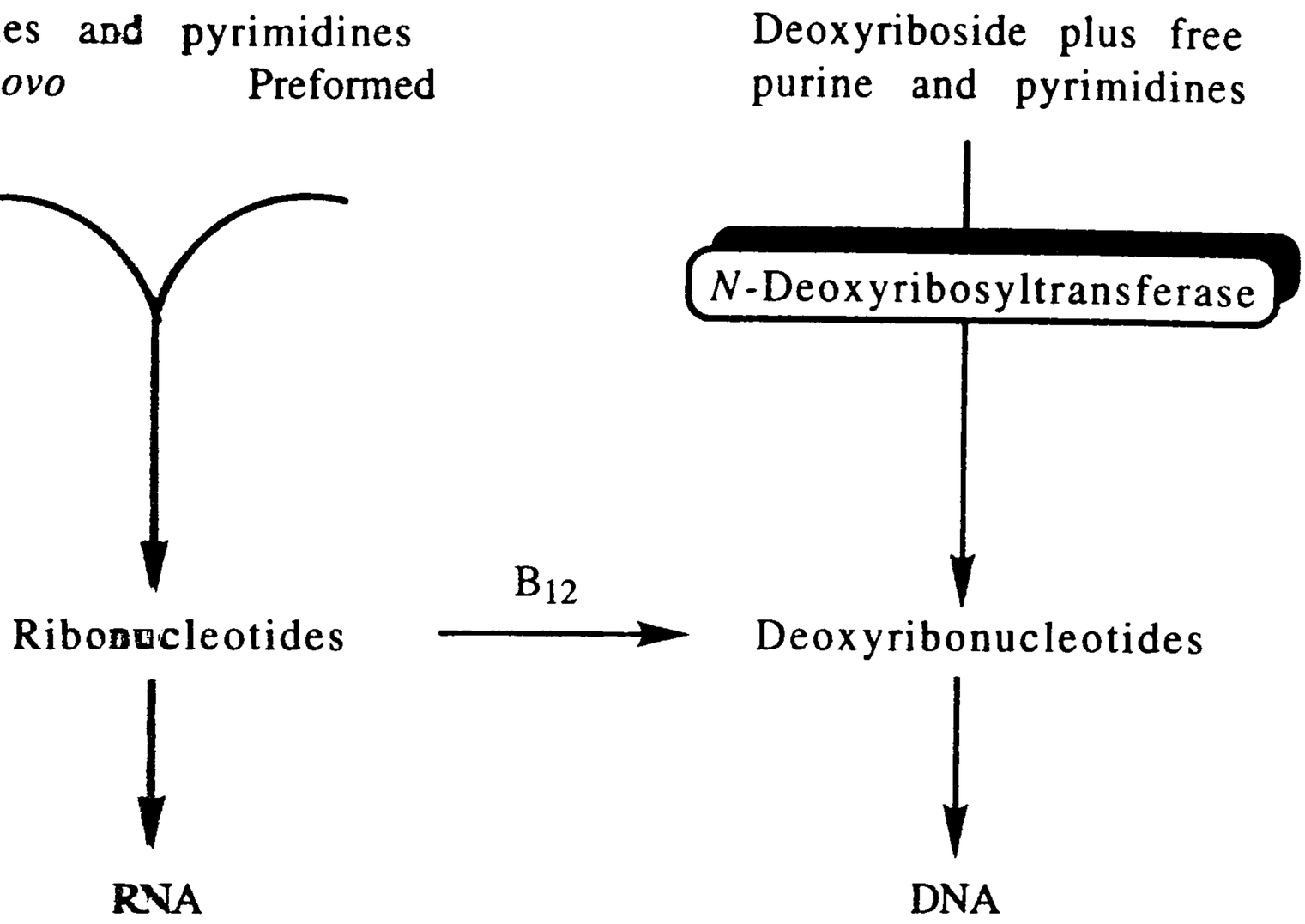


Fig. 1.6 Function of *N*-deoxyribosyltransferase in cells

N-Deoxyribosyltransferases in the Synthesis of Nucleoside Analogues

The *N*-deoxyribosyltransferases offer an alternative route to nucleoside phosphorylases for the synthesis of nucleoside analogues.[68] For the synthetic preparation of nucleosides with possible antiviral activity the transferases from *Lactobacillus helveticus* and *Lactobacillus leichmannii* are usually used as crude preparations without separation of the two discrete transferase activities.[107] This is a very convenient method as the minimum purification is required. As with the nucleoside phosphorylases, the *N*-deoxyribosyltransferase reaction is a very regioselective and stereoselective process; the glycosyl bond is formed at only one nitrogen within the heterocyclic base in a β-configuration.[108]

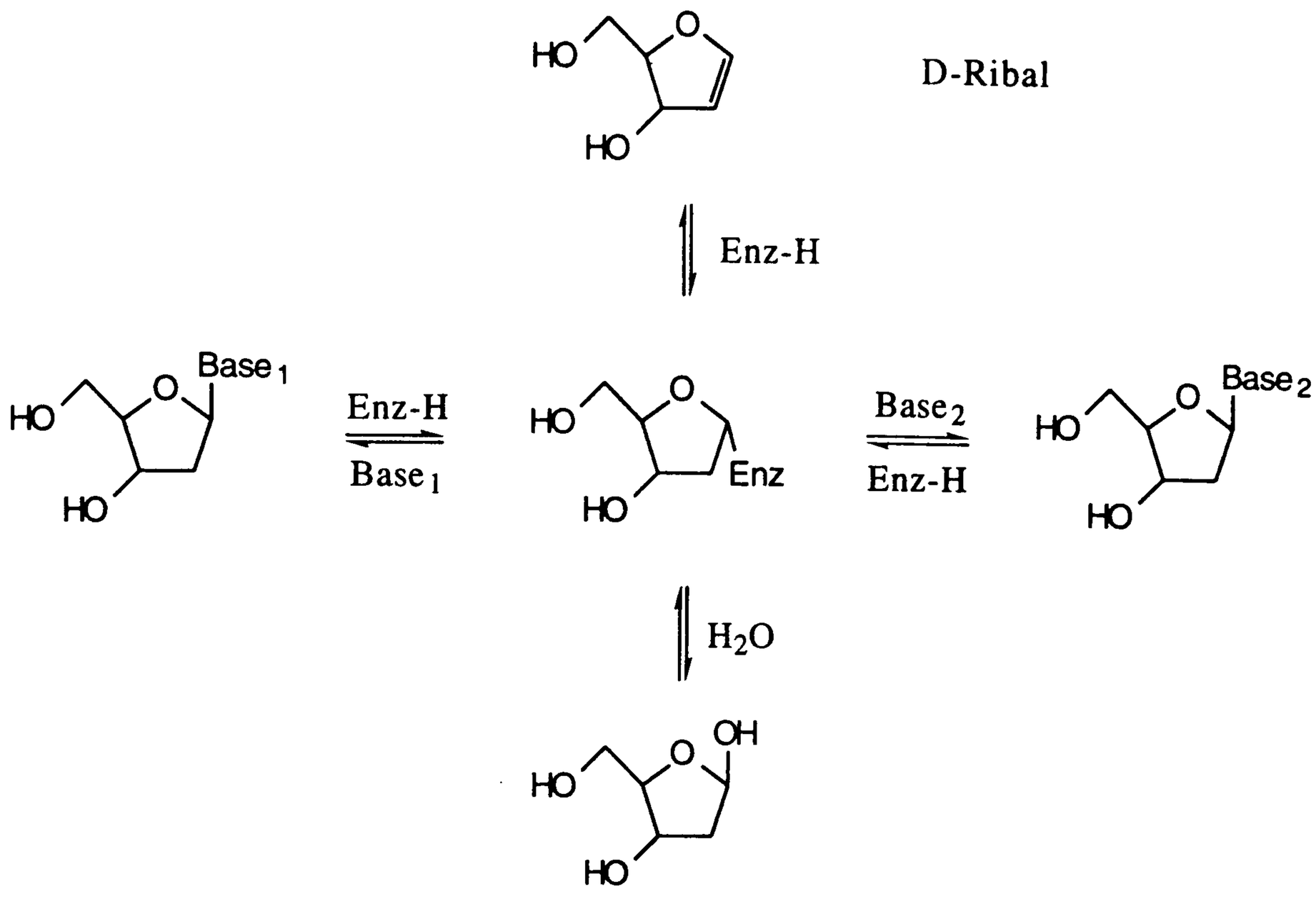
The use of crude enzyme preparations simplifies the synthetic procedure, but side reactions catalysed by contaminating hydrolytic and/or deamination activities in the crude enzyme preparation can occur when

long reactions times are used. Addition of water-miscible organic solvents to the reaction inhibit this degradation. It was found that the glycosyl transfer reaction was not effected if the concentration of the organic solvent was kept low. The best results were achieved by the addition of 10% (v/v) ethylene glycol to the reaction which inhibited any contaminating enzyme while allowing the transfer reaction to proceed as normal.[109]

In the transferase reactions, thymidine or 2'-deoxycytidine are the most effective glycosyl donors. The transferases will only accept 2'-deoxy-, 2',3'-dideoxy- and 2',5'-dideoxynucleosides as the donors: no other sugar modifications have yet been found which are tolerated. As with nucleoside phosphorylases a wide range of heterocyclic bases are accepted as substrates, as in the synthesis of 2'-deoxyadenosine[110] and imidazole-2'-deoxynucleoside[111] derivatives. The reaction is ideal for the preparation of radiolabelled compounds as the radiolabel is found exclusively in either the base or the sugar moiety of the product with no contamination in other parts of the nucleoside.[112, 113]

More recently the transferases have been used in the synthesis of 2',3'-dideoxynucleosides with antiviral activity.[112] A vast range of base modified 2',3'-dideoxynucleosides have now been synthesised by enzymatic means for screening as potential anti-HIV drugs.[65, 114-118]

Recently 1,4-anhydro-2-deoxy-D-erythro-pent-1-enitol (D-ribal), a glycal of ribose, was shown to be formed in the enzymatic reactions. In the absence of acceptor bases, the *N*-deoxyribosyltransferase catalyses the slow hydrolysis of the 2'-deoxynucleosides to generate spontaneously D-ribal which only disappears later as 2'-deoxynucleoside hydrolysis approaches completion (Scheme 1.16).[119]



Scheme 1.16 Mechanism of nucleoside and D-ribal formation by *N*-deoxyribosyltransferase

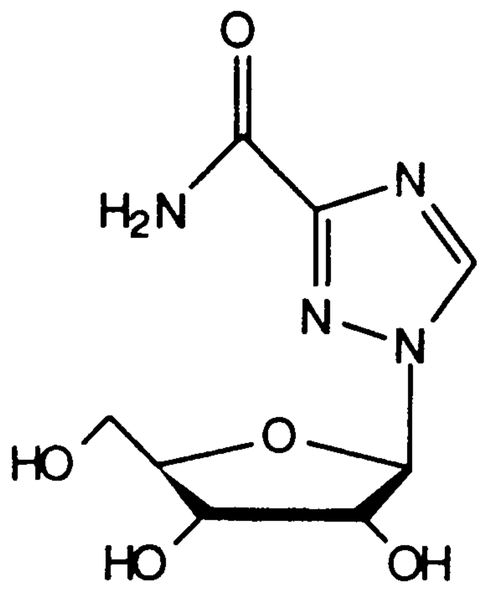
In the absence of heterocyclic bases, the *N*-deoxyribosyltransferase catalyses the hydration of D-ribal, but when the bases are present it catalyses the synthesis of 2'-deoxynucleosides. This new method eliminates the substrate nucleoside from the reaction mixture, facilitating purification, but as yet only the synthesis of natural substrates has been reported. The stereochemistry of the nucleoside formation from D-ribal supports previous evidence for the presence of a deoxyribosyl-enzyme intermediate.

To summarise, the *N*-deoxyribosyltransferases have practical application in the synthesis of a wide range of nucleoside analogues. The transferases

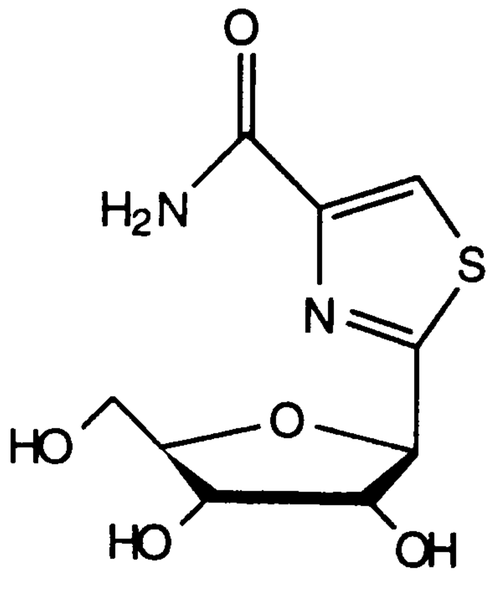
tolerate minor modification in the sugar moiety and a great variety of heterocyclic bases. The *N*-deoxyribosyltransferases have an advantage over nucleoside phosphorylases in that only one enzyme is required for the transfer of the glycosyl residue between purines and pyrimidines in the synthesis of a whole range of analogues, thus making it a more general and simpler method giving a cleaner reaction.

Triazole Nucleoside Analogues

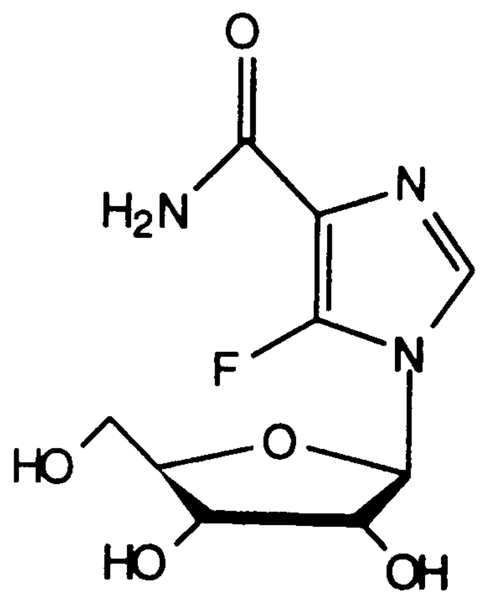
The success of ribavirin has stimulated the synthesis and antiviral testing of a large number of glycosides of related 5-membered heterocycles.[120, 25, 26, 43, 121, 120] Analogues (Fig. 1.7), such as, FICAR (5-fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide), tiazofurin, EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide)[122] and selenazofurin[123], these, along with other 5-halogenated and 5-alkynyl imidazole-4-carboxamides,[124] are all active against orthomyxoviruses, paramyxoviruses and arenaviruses.



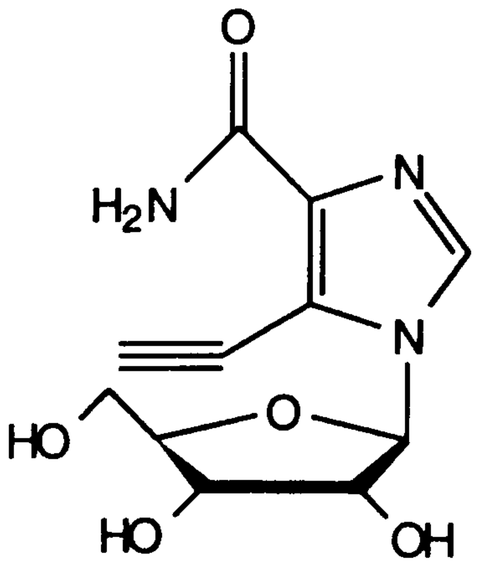
Ribavirin



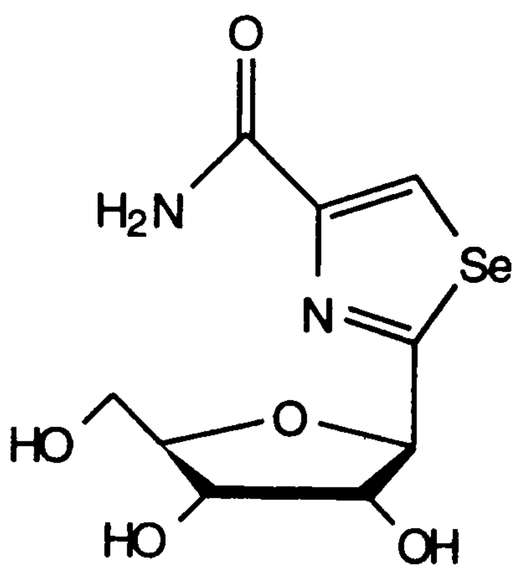
Tiazofurin



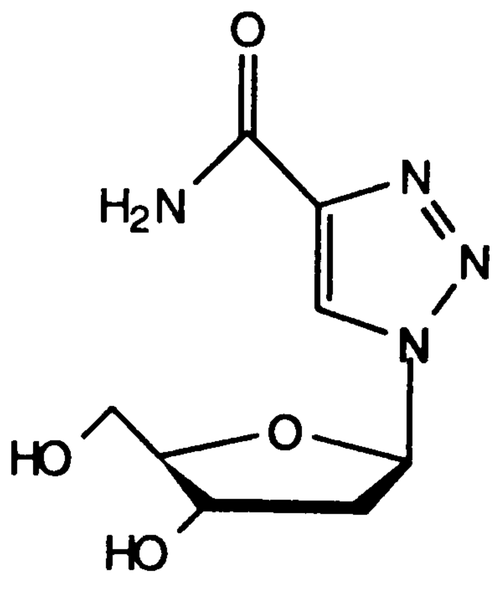
FICAR



EICAR



Selenazofurin



8

Fig. 1.7 Analogues of ribavirin

Various 1,2,3-triazole nucleoside analogues have been synthesised, most show little antiviral activity.[59, 125-130] 1-β-D-2'-Deoxyribofuranosyl-1,2,3-triazole-4-carboxamide **8** has been reported to have antiviral activity.[57] A range of 1,2,4-triazole nucleosides with sugars other than D-ribose, α – and β-arabinofuranosyl,[58, 131] various thio-,[132] fluoro-,[133] deoxy- and dideoxyribofuranosyl derivatives,[134, 135] and other sugars[136-139] have been synthesised. The majority show little antiviral activity. 2'-Deoxyribavirin has been patented as antiviral[140] though Witkowski *et al*[58] reported it as inactive. 2',3'-Dideoxyribavirin again shows no activity against the HIV retrovirus,[135] but is patented as a virucidal agent.[118] The 2',3',5'-tri-*O*-acetate of ribavirin has been prepared as a lipophilic prodrug and appears to cross the blood brain barrier.[141] Any modification of the β-

D-ribofuranosyl moiety other than phosphorylation or acetylation leads to loss of activity.[26] The heterocycle of ribavirin, 1,2,4-triazole-3-carboxamide (TCA), is reported to have antiviral activity. The base is converted enzymatically to ribavirin *in vivo*.[142] The carboxamidine of ribavirin is active against both DNA and RNA viruses, but the thiocarboxamide is only active against DNA viruses.[55] Other disubstituted carboxamidine ribavirin derivatives were found to inhibit nucleoside phosphorylases.[56] Any other changes in the carbamoyl group reduced or lost the antiviral activity of the nucleoside.[12, 55, 143, 144] Structure activity analysis[145] confirms that the carboxamide group,[146] the 1,2,4-triazole ring,[147] and the β-D-ribofuranosyl moiety are essential for antiviral activity. Theoretical studies on the conformation of ribavirin have been reported (Fig. 1.8).[145] Ribavirin crystallises in two polymorphic forms, V1 and V2.[33] V1 exhibits a glycosyl torsional angle χ(O(1')-C(1')-N(1)-C(5)) of 10.4° denoted as high *syn* and the ribose conformation is 3'*endo*-2'*exo*. V2 has a χ value of 119.0° referred to as high *anti* and a 2'-*exo*-1'-*endo* ribose conformation (Fig. 1.8). Ribavirin exists in solution in the high *syn* conformation, but this was considered not to be the active form of the drug. The inactive 5-methyl and 5-chloro derivatives,[148] which also exist in the high *syn* forms, lack an energy minimum in the high *anti* region. Ribavirin does have a second minimum energy corresponding to the high *anti* conformation, and it has been suggested that the active conformation of ribavirin at the enzyme site is the high *anti* form (Fig. 1.8).

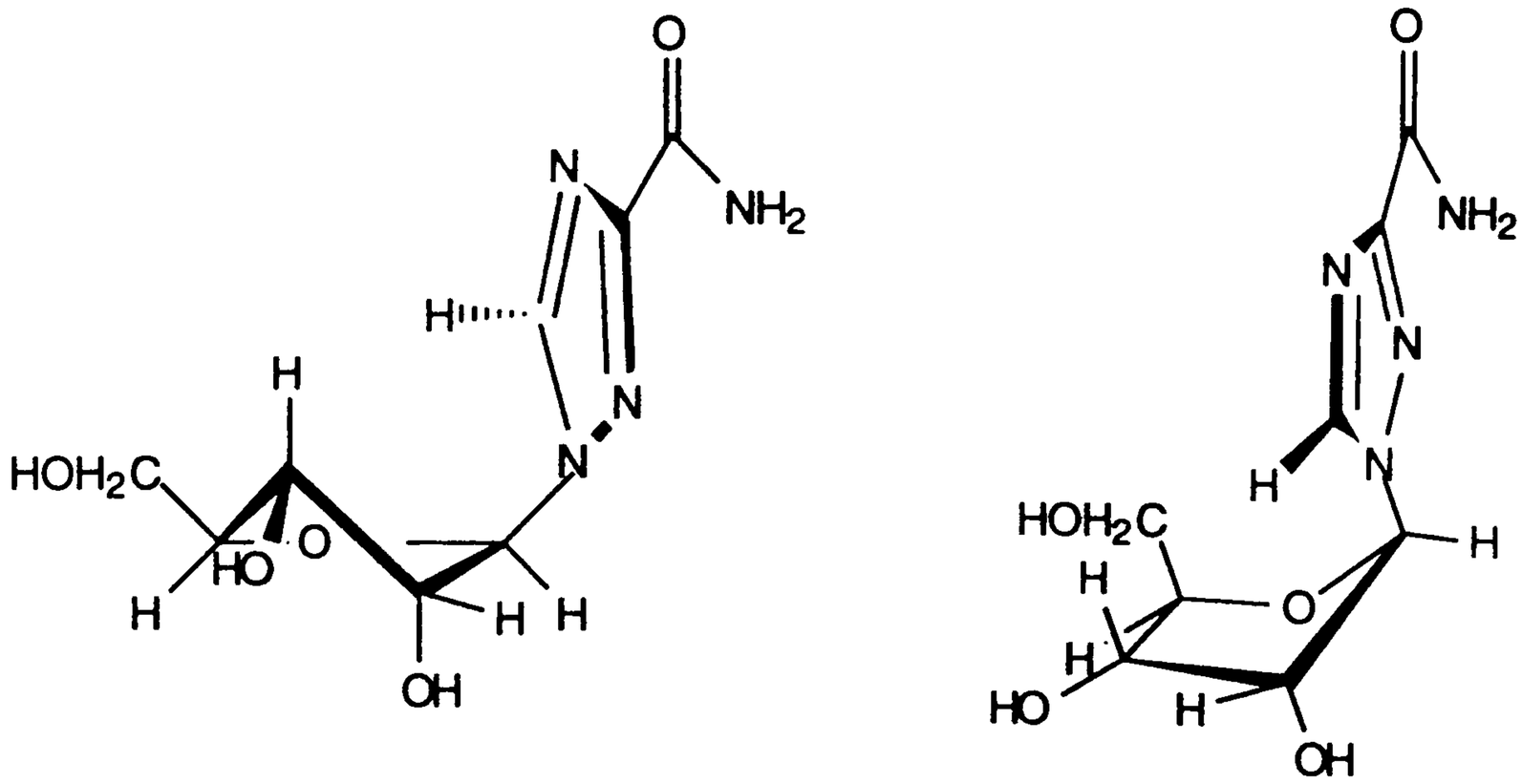
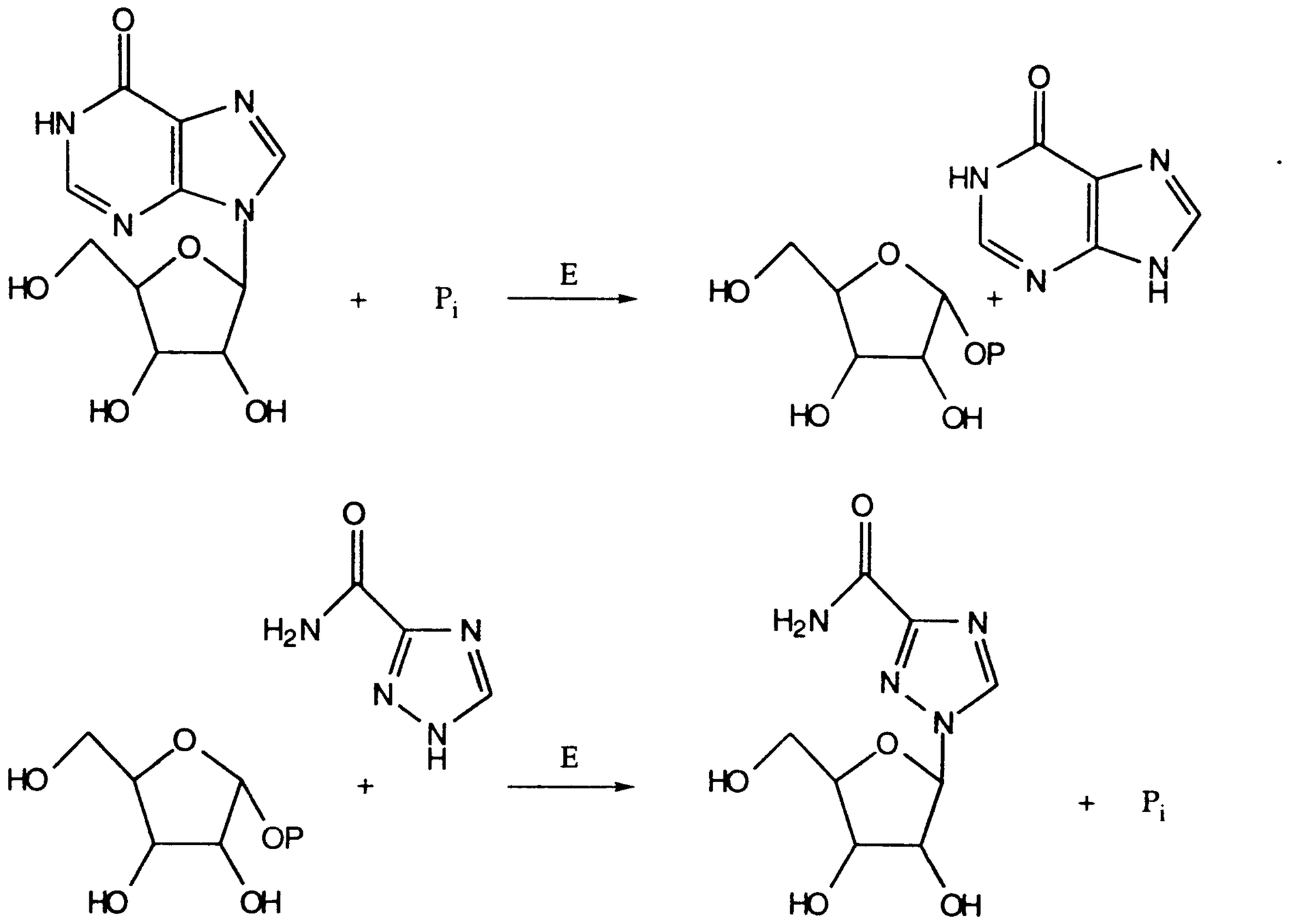


Fig. 1.8 Two conformations of ribavirin

Enzymatic Synthesis of Triazole Nucleoside Analogues

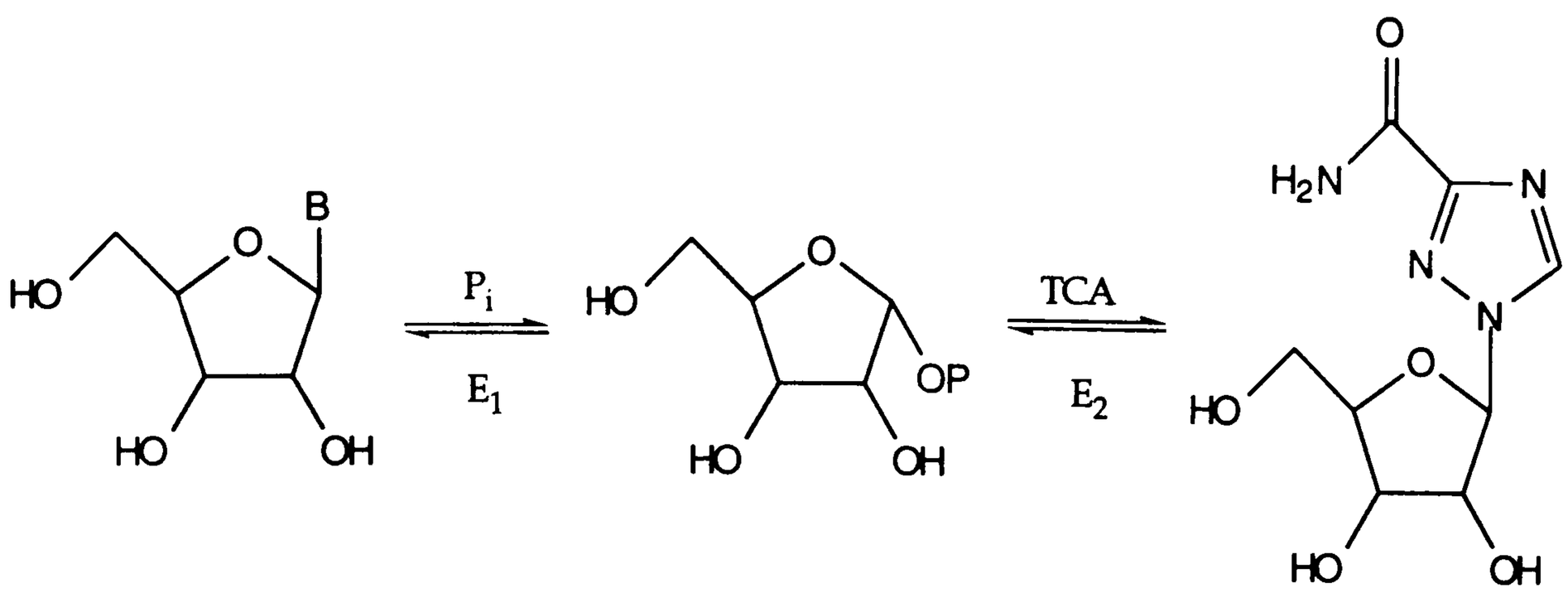
The enzymatic synthesis of ribavirin was first reported by Ochiai *et al*[149] in 1976 from a whole cell culture of mutant *Bacillus subtilis* KY 4540, but the productivity was low. A two step reaction using purified purine nucleoside phosphorylase from *Enterobacter aerogenes* AJ 11125 has been used to synthesise ribavirin where the glycosyl donor was inosine (Scheme 1.17). The initial step involved the production of ribose-1-phosphate from the enzymatic phosphorolysis of the purine nucleoside.[150] The ribose-1-phosphate was isolated by ion exchange chromatography before it was combined with 1,2,4-triazole-3-carboxamide (TCA). A transribosylation reaction then produced the nucleoside virazole. TCA has a low affinity for the purine nucleoside phosphorylase as compared to that of the hypoxanthine base. If the hypoxanthine base is retained in the reaction, it competes with TCA for the enzyme.



E = nucleoside phosphorylases from *Enterobacter aerogenes* AJ 11125

Scheme 1.17 Two step synthesis of ribavirin with nucleoside phosphorylases[150]

Shirae and co-workers have carried out an extensive screening programme to find microorganisms that produce ribavirin directly from TCA and pyrimidine nucleosides[151, 152] or TCA and purine nucleosides by whole cell culture and thus ribose-1-phosphate need not be isolated (Scheme 1.18).[153, 154] One of each type was purified and characterised.



B = purine or pyrimidine base

E_1, E_2 = nucleoside phosphorylases

TCA = 1,2,4-triazole-3-carboxamide

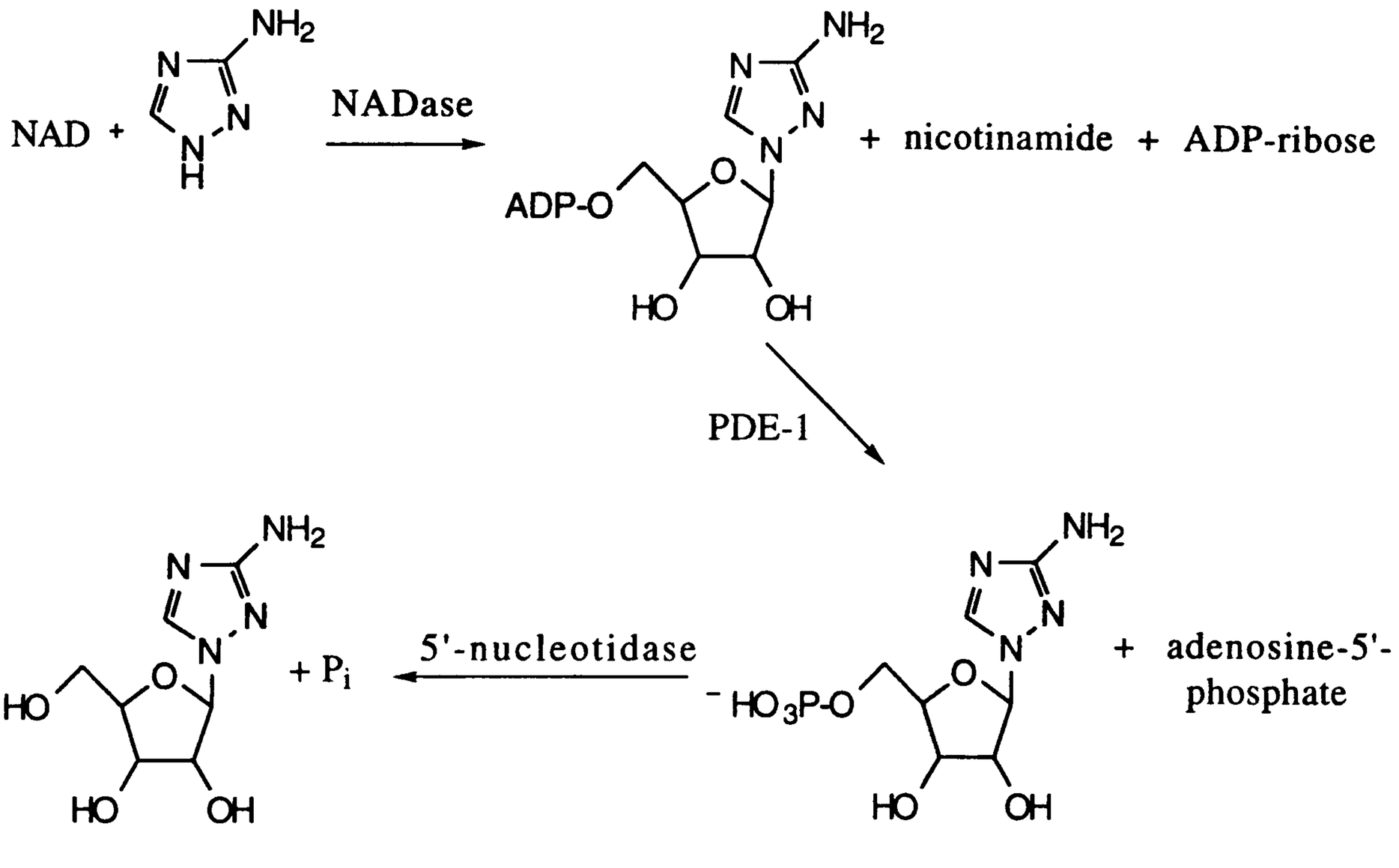
Scheme 1.18 Direct one-pot synthesis of ribavirin using nucleoside phosphorylases

The purine nucleoside phosphorylase from *Brevibacterium acetylium* ATCC 954 was purified and ribavirin was synthesised directly from TCA and guanosine or inosine.[155] The uridine/oritidine nucleoside phosphorylase and the pyrimidine nucleoside phosphorylase activities from *Erwinia cartovora* AJ 2992 were purified.[156] The orotidine phosphorylating enzyme could hydrolyse orotidine but could not produce ribavirin in the presence of TCA. Likewise, the purine nucleoside phosphorylase could not split orotidine. However, the synthesis of ribavirin from TCA and oritidine was possible when both enzymes were used. Thus, both enzyme catalysed reactions are irreversible and, together, give good yields of ribavirin. A combination of microorganisms was used to increase the productivity of the whole cell ribavirin reaction.[157] The enzymes from *Erwinia carotovora* AJ 2992 catalysed the phosphorolysis of inosine, and those from *Bacillus brevis* the ribosylation of TCA. This proved to be a more efficient and productive system than the one using whole cells of *Enterobacter*

aerogenes AJ 11125 alone.

The syntheses of ribavirin,[158-160] 2'-deoxyribavirin[140, 161] and 2',3'-dideoxyribavirin[118] with nucleoside phosphorylases have been reported.

A range of 1,2,4-triazole adenine dinucleotides has been made by the nicotinamide adenine dinucleosidase (NAD nucleosidase) catalysed trans-adenosinediphosphate-ribosylation with nicotinamide adenine dinucleotide (NAD). Treatment with phosphodiesterase (PDE-1) and 5'-nucleotidase gave the corresponding mononucleoside (Scheme 1.19).[162] All the 3-substituted 1,2,4-triazole bases gave the N-1 substituted product exclusively. The parent 1,2,4-triazole base gave the N-1 and N-4 ribosylated product.



NAD = nicotinamide adenine dinucleotide

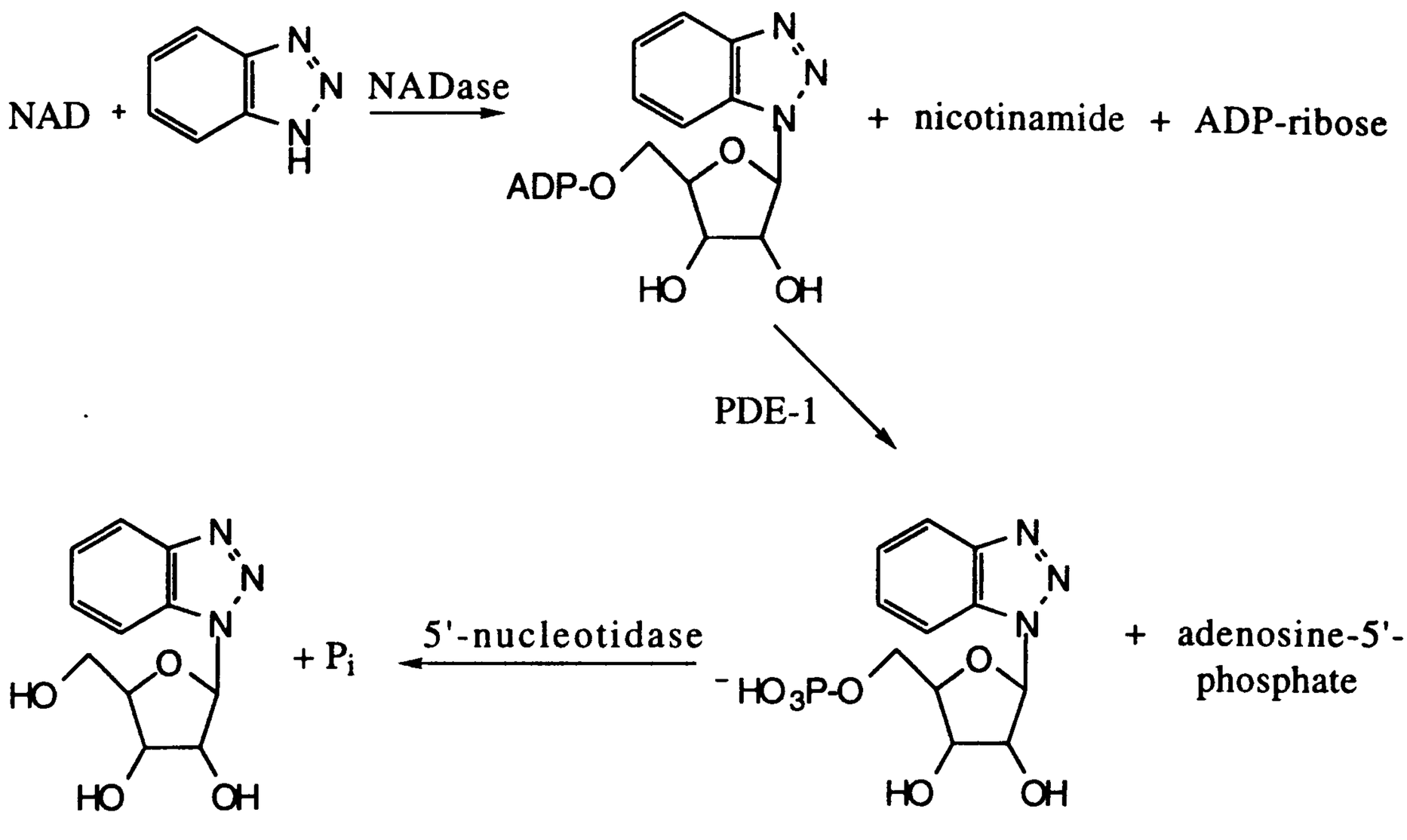
NADase = nicotinamide adenine dinucleosidase

ADP = adenosine diphosphate

PDE-1 = phosphodiesterase

Scheme 1.19 Synthesis of ribavirin analogues with NADase

Previous to this, a number of benzotriazole and related triazoles were ADP ribosylated using a crude enzyme extract of porcine brain NADase by the same methodology (Scheme 1.20).[163] In all cases only the N-1 ribosylated product was formed.



NAD = nicotinamide adenine dinucleotide

NADase = nicotinamide adenine dinucleosidase

ADP = adenosine diphosphate

PDE-1 = phosphodiesterase

Scheme 1.20 The synthesis of benzotriazole nucleosides with NADase

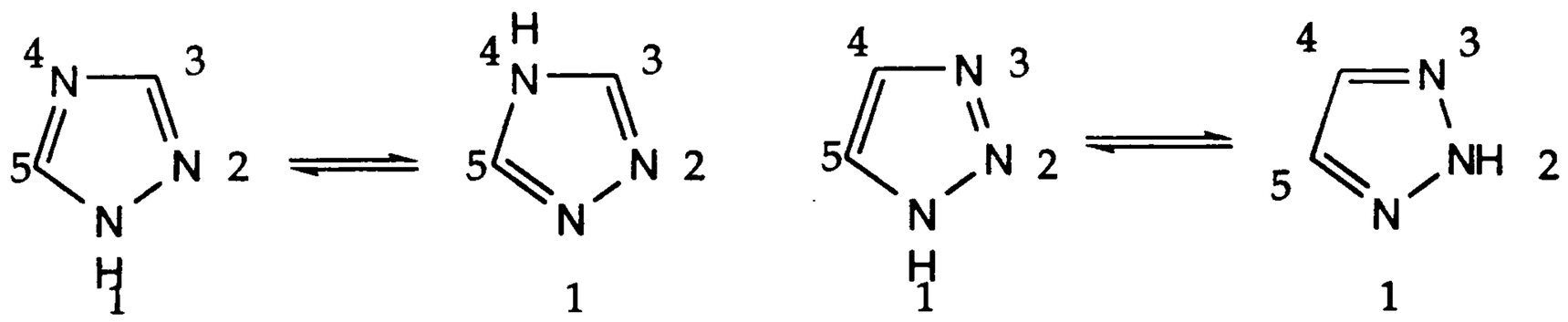
The synthesis of nucleoside analogues of ribavirin using *N*-deoxyribosyltransferases has yet to be reported.

CHAPTER 2

SYNTHESIS OF TRIAZOLE BASES

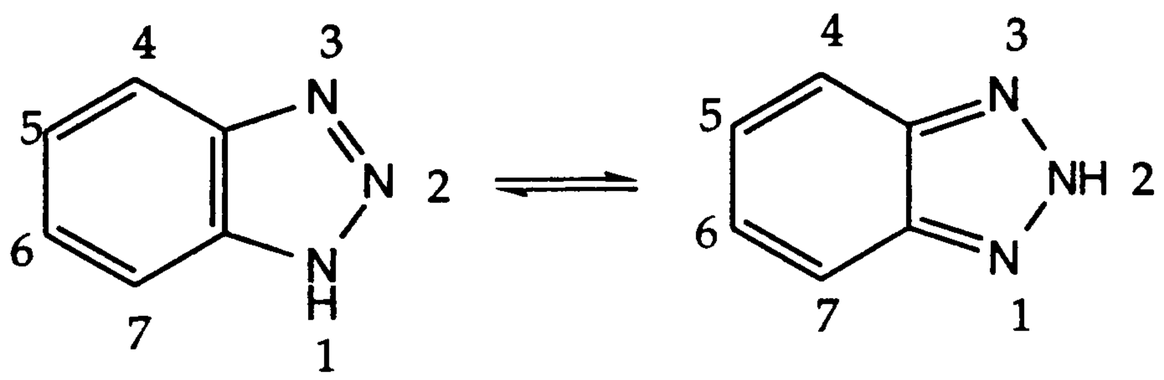
Background

The discovery of ribavirin with its broad spectrum of antiviral activity has stimulated the synthesis of a wide range of 1,2,3-triazole and 1,2,4-triazole nucleoside analogues. Ribavirin and a few ribavirin analogues have been made using nucleoside phosphorylases and NAD nucleosidase, as already mentioned in Chapter One, but no synthesis using *N*-deoxyribosyltransferase has yet been reported. The parent compounds 1,2,4-triazole and benzotriazole (Fig. 2.1) are known to act as acceptors in the transglycosylation reaction catalysed by *N*-deoxyribosyltransferase from *Lactobacillus leichmannii*.[98]



1,2,4-Triazole ⇌ 1,2,4-Triazole

1,2,3-Triazole ⇌ 1,2,3-Triazole



Benzotriazole ⇌ Benzotriazole

Fig. 2.1 The structures of the parent triazole bases

In this work, series of triazoles were synthesised as base substrates for the enzymatic synthesis of triazole nucleoside analogues. All the triazoles were synthesised by methods published in the literature. All but one of the *N*-substituted triazole carboxamides are new compounds.

Results and Discussion

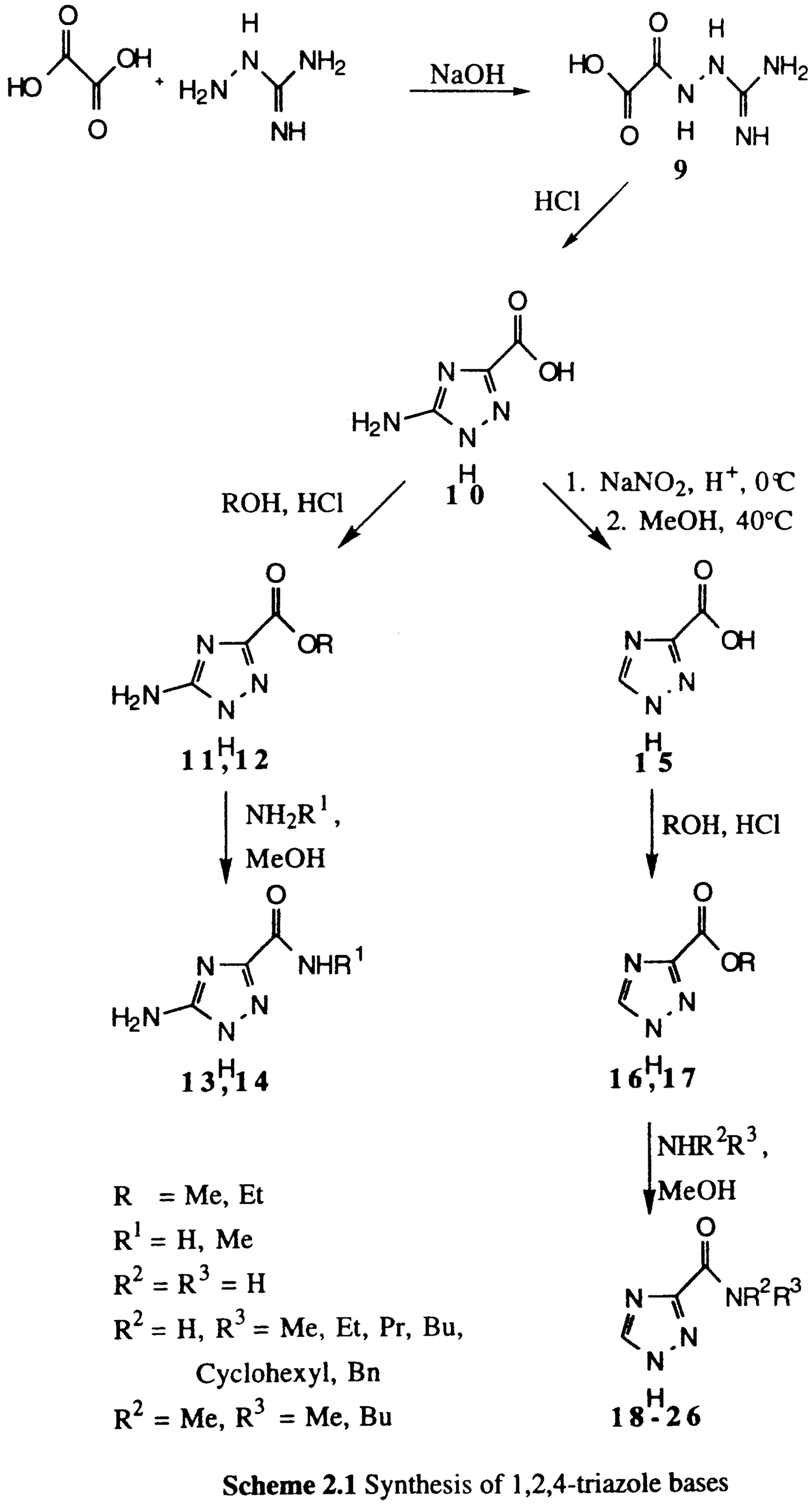
Synthesis of 1,2,4-Triazole Bases

All of the 1,2,4-triazole derivatives were synthesised from the parent compound 5-amino-1,2,4-triazole-3-carboxylate **10** (Scheme 2.1). This was made from aminoguanidine bicarbonate and oxalic acid *via* the oxalic acid monoguanylhydrazide **9** in almost quantitative yield.[164, 165] However, 5-amino-1,2,4-triazole-3-carboxylic acid is now commercially available. Treatment of the triazole **10** with sodium nitrite in acid[166] gave the diazonium salt, which is explosive when dry. The damp solid was suspended in methanol and heated to 40°C, when an exothermic reaction occurred yielding 1,2,4-triazole-3-carboxylate **15**. The impure acid was used for the esterification, as recrystallisation from water caused decarboxylation reducing the yield.

From 5-amino-1,2,4-triazole-3-carboxylate **10** and 1,2,4-triazole-3-carboxylate **15** a range of 1,2,4-triazole esters and carboxamides were made (Scheme 2.1) (Table 2.1). The methyl **11, 16** and ethyl esters **12, 17** were synthesised by esterifying the acid at room temperature in the appropriate alcohol saturated at 0°C with hydrogen chloride gas.[164, 166, 167]

The 1,2,4-triazole-3-carboxamides were synthesised by dissolving the triazole esters neat in the required amine, sometimes methanol was added to homogenise the solution and left to react at room temperature or 50°C

(Scheme 2.1) (Table 2.1). Reactions at the lower temperature were slower but were easier to purify by recrystallisation.



Scheme 2.1 Synthesis of 1,2,4-triazole bases

The reactions were followed by reverse phase HPLC on a Techsphere 5C8

or Techsphere 10ODS column (25 cm x 4.6 mm and a precolumn, 5 cm x 4.6 mm), using a mobile phase of acetonitrile and double distilled water (5:95, v/v) at a flow rate of 1.0 ml/min with detection by UV at 214 nm. When the reactions were completed, the solvent was removed and in most cases all that was required for purification was recrystallisation.

Typical ^{1}H NMR and ^{13}C NMR spectral data of triazoles are shown in Fig. 2.2:

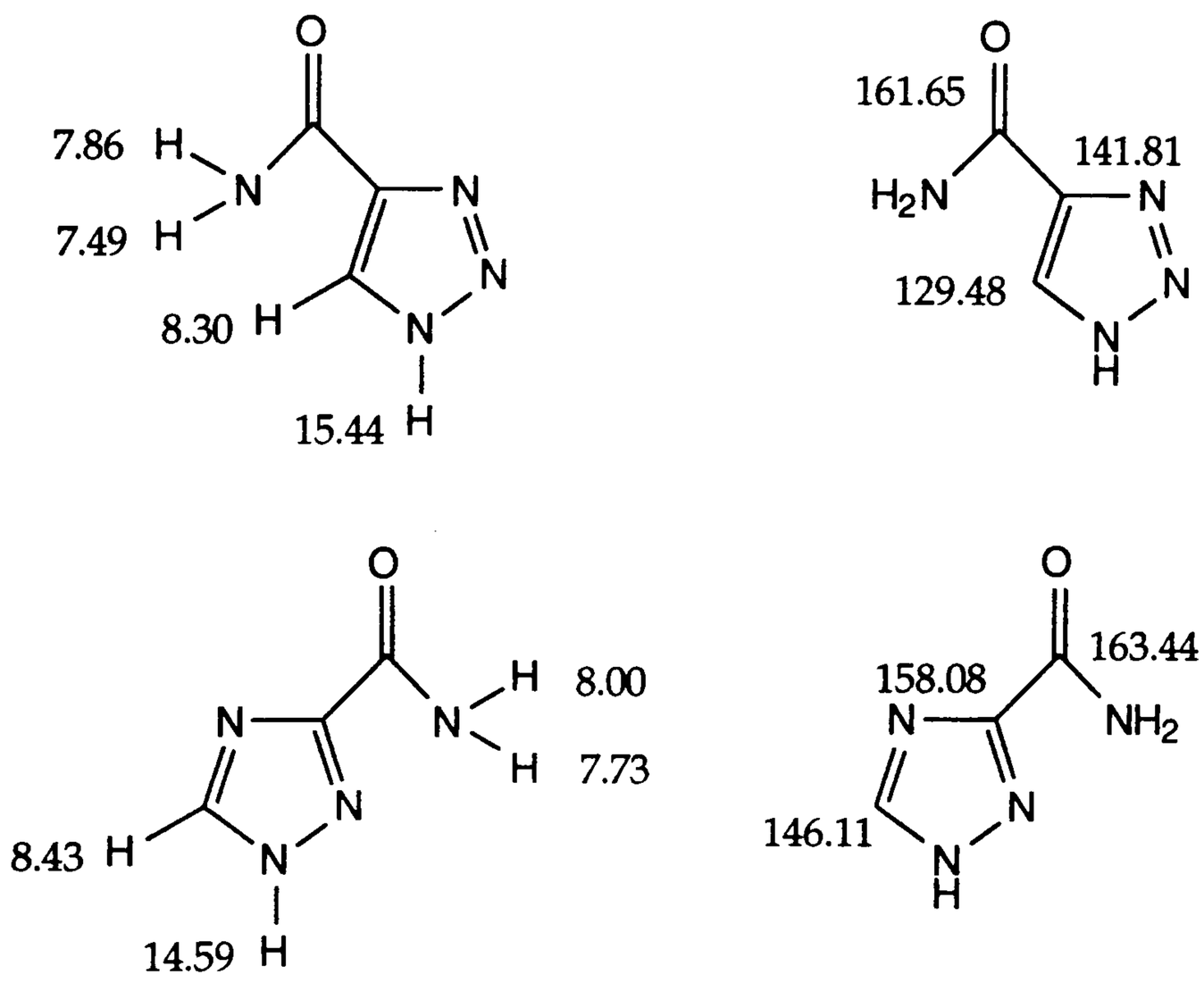
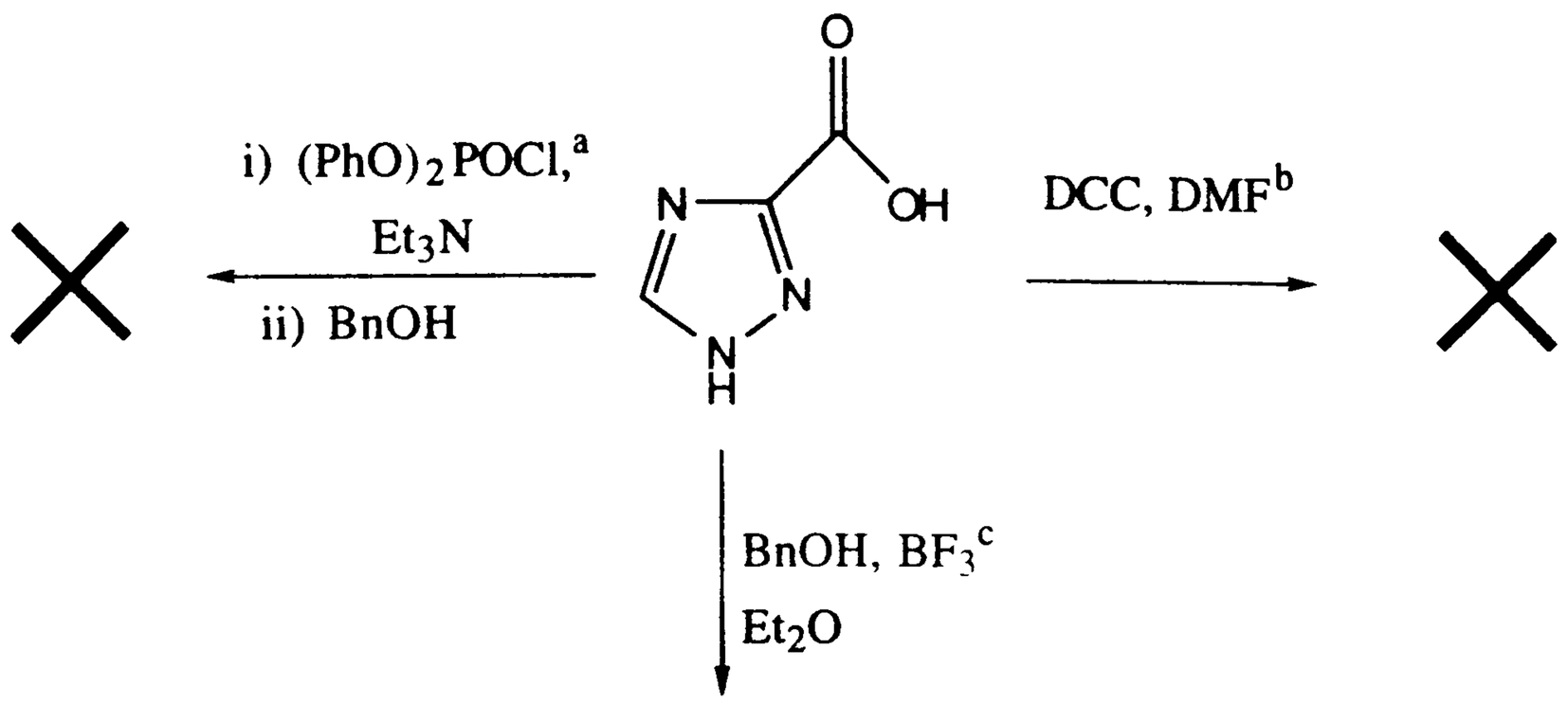


Fig 2.2 ^{1}H and ^{13}C NMR data for 1,2,3-triazole-4-carboxamide and 1,2,4-triazole-3-carboxamide

One of the difficulties encountered at the beginning of this investigation was the detection and characterisation of the triazoles synthesised. Most only absorb at low UV wavelengths, typically 220 nm, and have few protons for ^{1}H NMR analysis (Fig. 2.2). A range of thin layer chromatography (TLC) sprays were screened to find a generally applicable, sensitive spray reagent for the triazole ring, but without

success.[168-172]

Accordingly, attempts were made to synthesise the unknown benzyl 1,2,4-triazole-3-carboxylate **27**, use of which would solve the dual problems of solubility and detection. The benzyl ester group would increase the solubility of the triazole in organic solvents. In addition, it would also be a good marker as it is UV-active at 254 nm and its disappearance in the amidation reactions could be monitored by TLC. Classical methods, employing dicyclocarbodiimide in dimethylformamide, benzenesulfonic acid or boron trifluoride as catalysts, formation of the acyl chloride using thionyl chloride or oxalyl chloride or diphenylphosphoryl chloride gave no detectable product (Scheme 2.2 and 2.3). The problems of solubility, even in dimethylformamide and dimethylsulfoxide and the known ease with which triazole carboxylic acids undergo decarboxylation[173] could account for these failures. Difficulties in using thionyl chloride have been reported,[174] where sulfur containing triazole products were isolated. This was avoided by using oxalyl chloride to form the acid chloride instead. Unfortunately, on repeating this procedure no benzyl 1,2,4-triazole-3-carboxylate was formed.

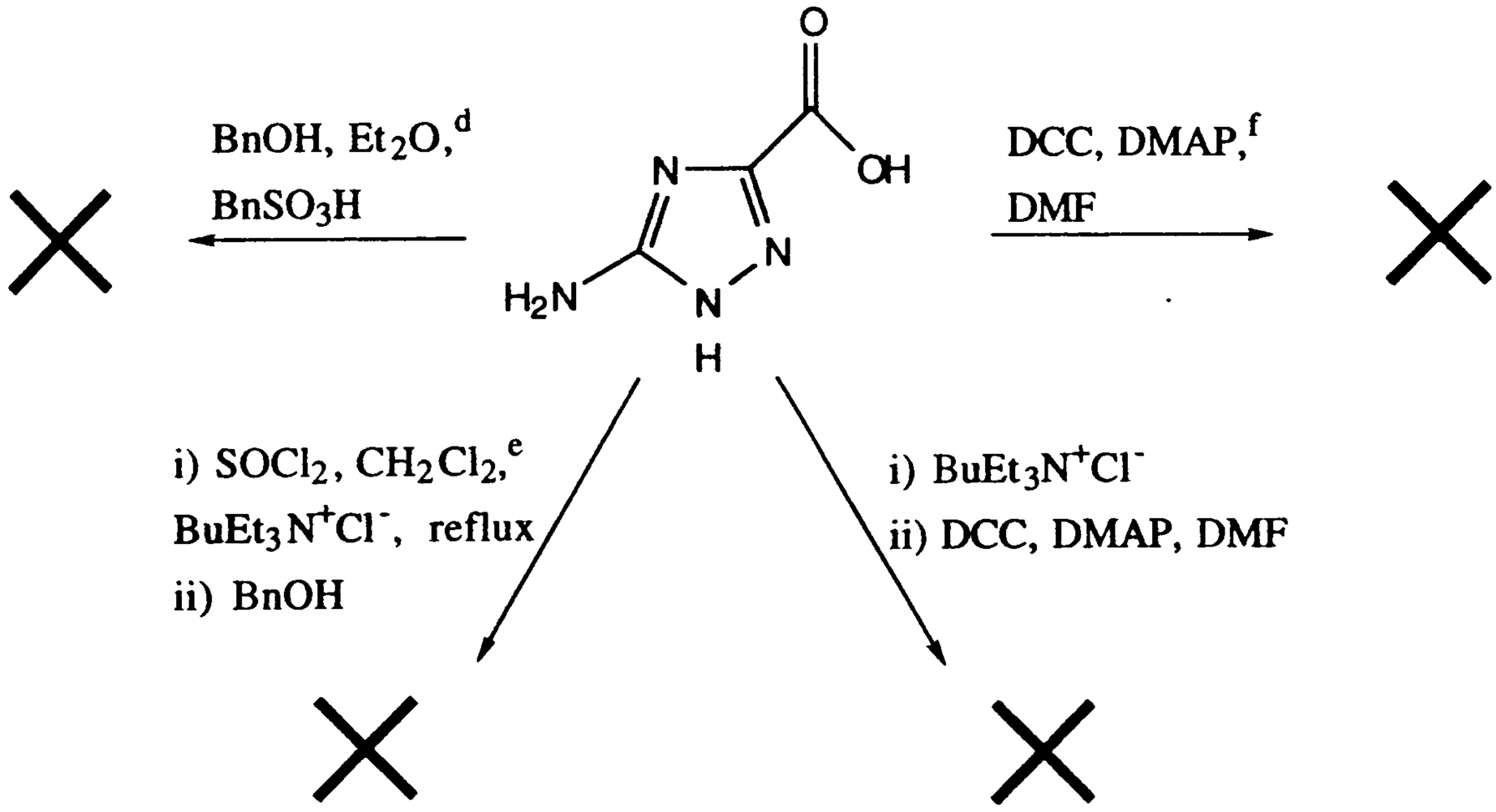


References: a[175]

b[176]

c[177]

Scheme 2.2 Procedures tried in the synthesis of benzyl 1,2,4-triazole-3-carboxylate



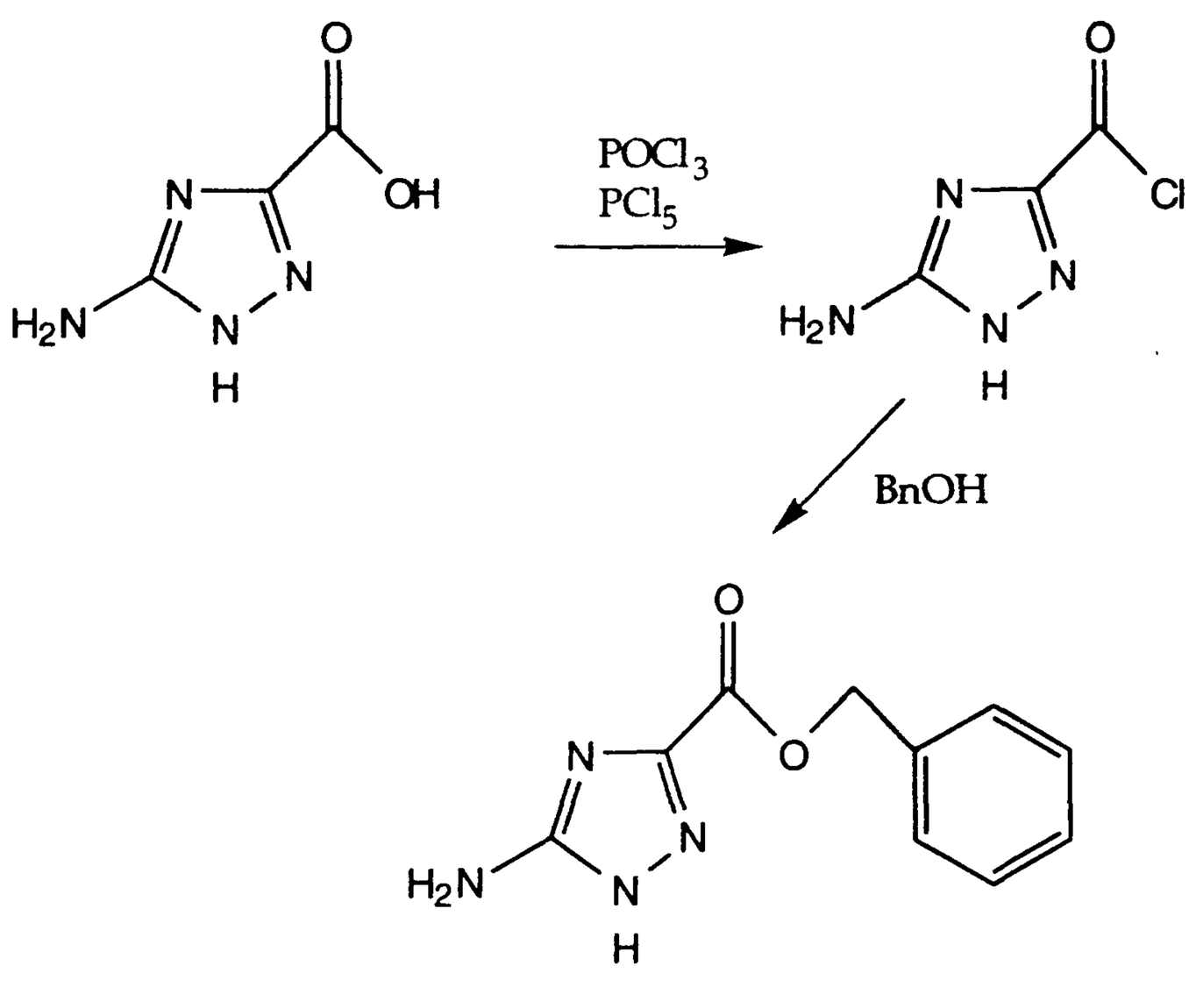
References: d[178]

e[179]

f[180]

Scheme 2.3 Procedures tried in the synthesis of benzyl 5-amino-1,2,4-triazole-3-carboxylate

Finally, using $POCl_3$ and PCl_5[181] a small amount of the desired ester **27** was formed, but in poor yield (Scheme 2.4). Investigation of this synthesis was discontinued as the other method, using the ethyl or methyl ester in the amidation reaction and following the reaction by HPLC, was found to be satisfactory.



27

Scheme 2.4 Synthesis of benzyl 5-amino-1,2,4-triazole-3-carboxylate[181]

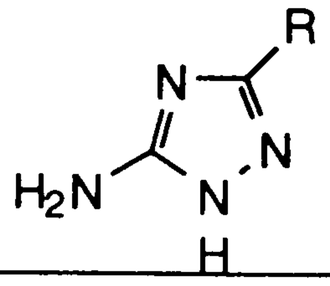
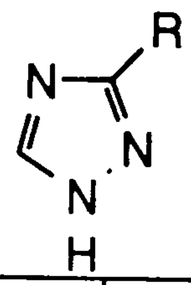
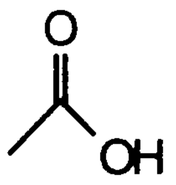
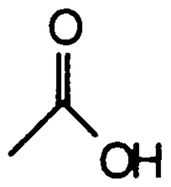
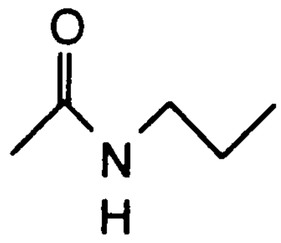
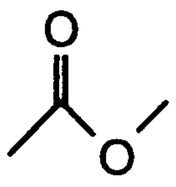
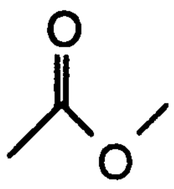
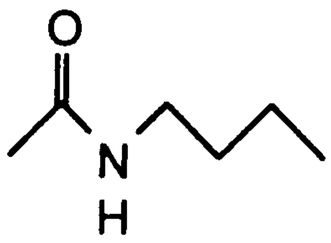
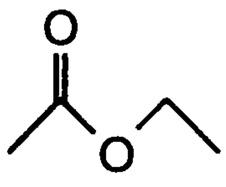
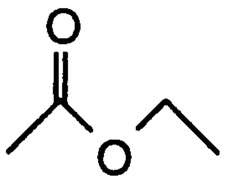
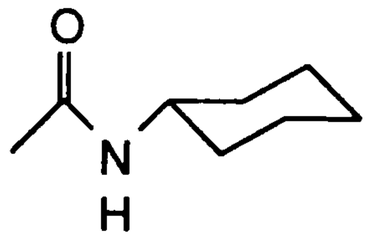
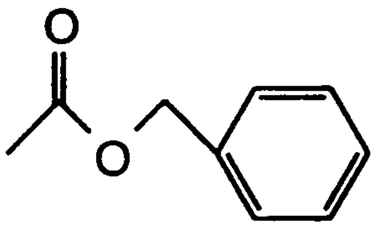
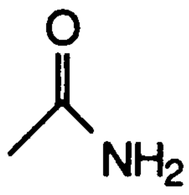
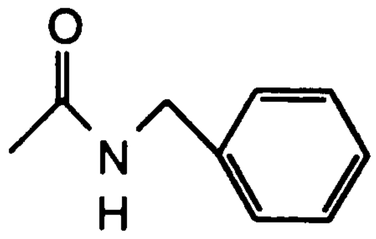
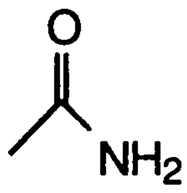
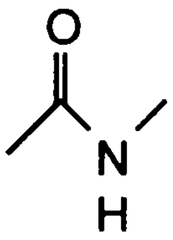
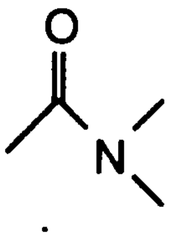
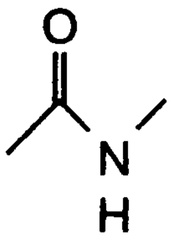
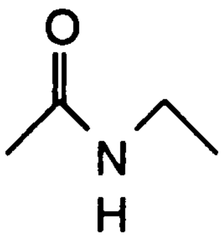
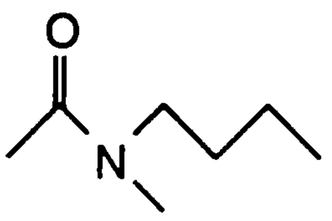
									
	R=	Yield %		R=	Yield %		R=		Yield %
10		98	**15**		94	**21**			72
11		78	**16**		89	**22**			79
12		53	**17**		50	**23**			37
27		15	**18**		94	**24**			25
13		90	**19**		81	**25**			68
14		97	**20**		92	**26**			40

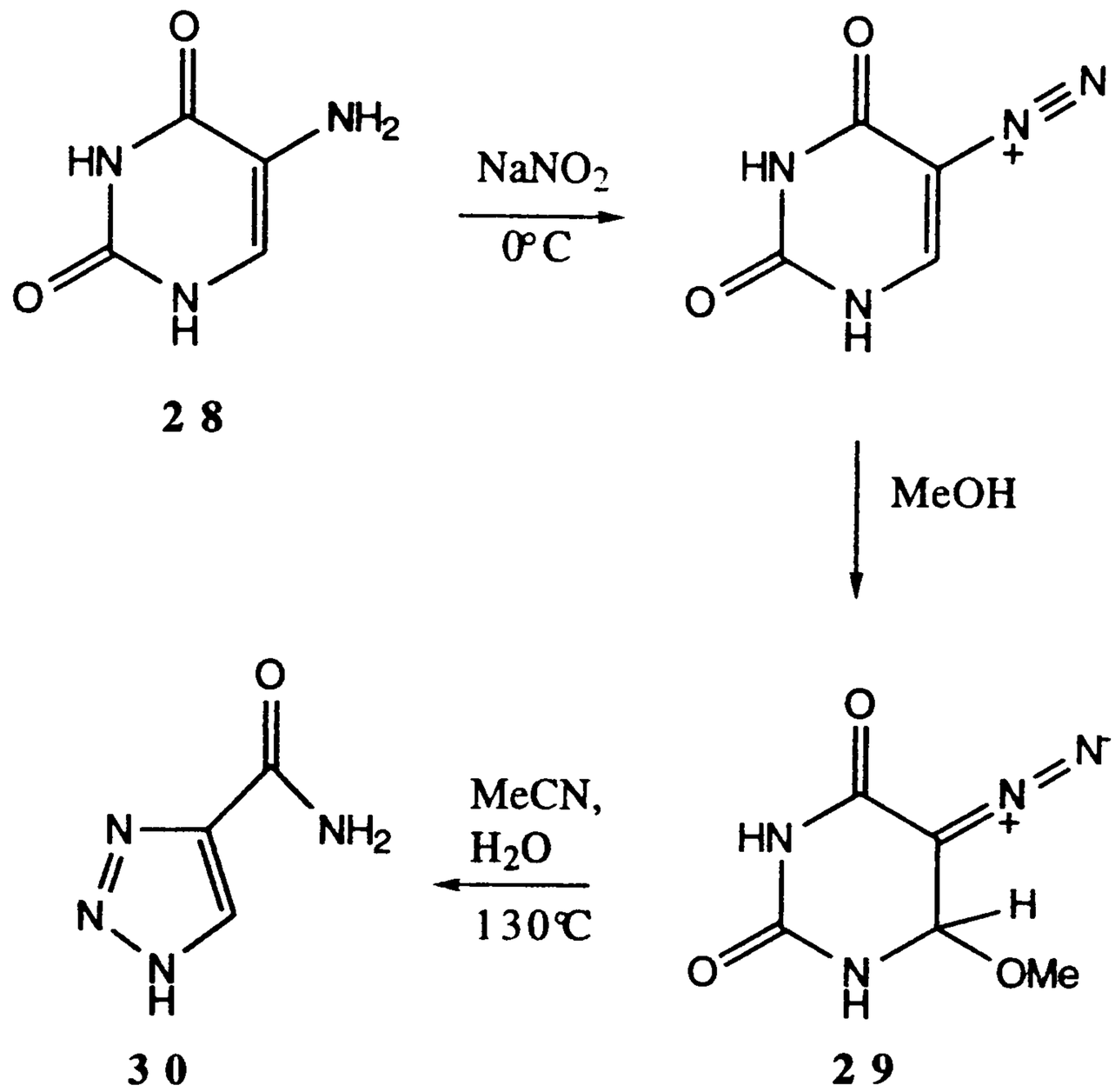
Table 2.1 1,2,4-Triazoles synthesised

Synthesis of 1,2,3-Triazole Bases

Synthesis of 1,2,3-Triazole-4-carboxamide

Two previously published approaches were investigated for the synthesis of 4-monosubstituted-1,2,3-triazoles. In the first, a novel ring contraction of 5-diazouracil to 1,2,3-triazole-4-carboxamide reported by Thurber *et al* was tried (Scheme 2.5).[182-184] In this synthesis, which was repeated exactly as published, 5-aminouracil **28** is diazotised and recrystallised from

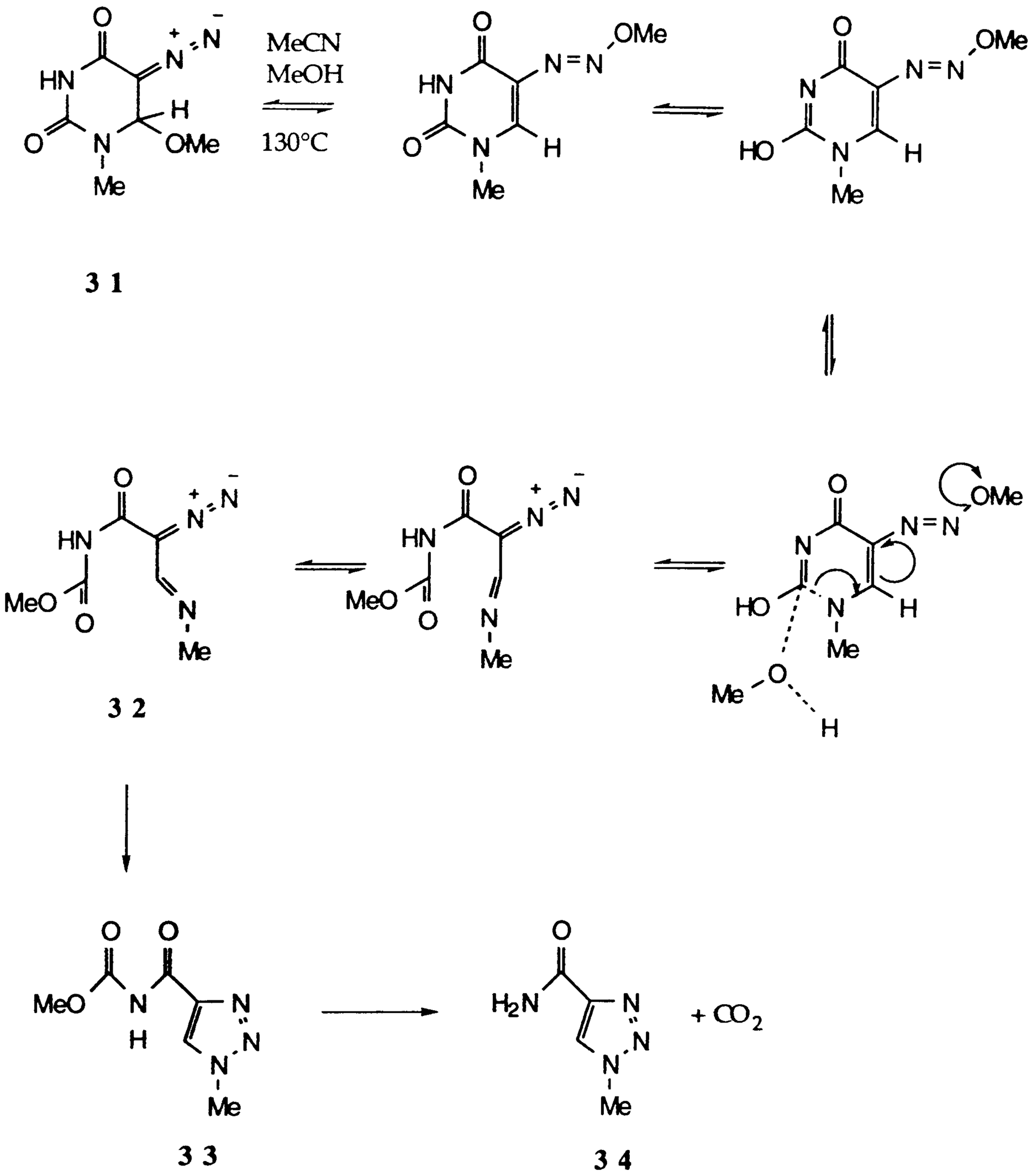
methanol. The methanol adduct **29** formed can be stored at room temperature indefinitely.



28 **30** **29**

Scheme 2.5 Synthesis of 1,2,3-triazole-4-carboxamide from 5-aminouracil[182, 184]

When the diazo compound **29** is heated in a sealed vessel at 130°C in a solution of 5% aqueous acetonitrile it undergoes hydrolysis with ring opening. This is followed by ring contraction and loss of carbon dioxide. Using ^{18}O labelling studies[184] Thurber *et al* showed that the ring contraction occurred without the exchange of the C-2 oxygen atom. No detectable traces of triazole were found if the proton on N-3 was replaced by a methyl group. In 5% methanolic acetonitrile the N-carbamate **33** of **30** was isolated. The following mechanism was proposed to explain these observations (Scheme 2.6) on the cyclisation of 5-diazo-1-methyluracil-6-methanolate in 5% methanolic acetonitrile **31** (Scheme 2.6).[184] The cyclisation of **32** has precedence in the literature.[185-187]



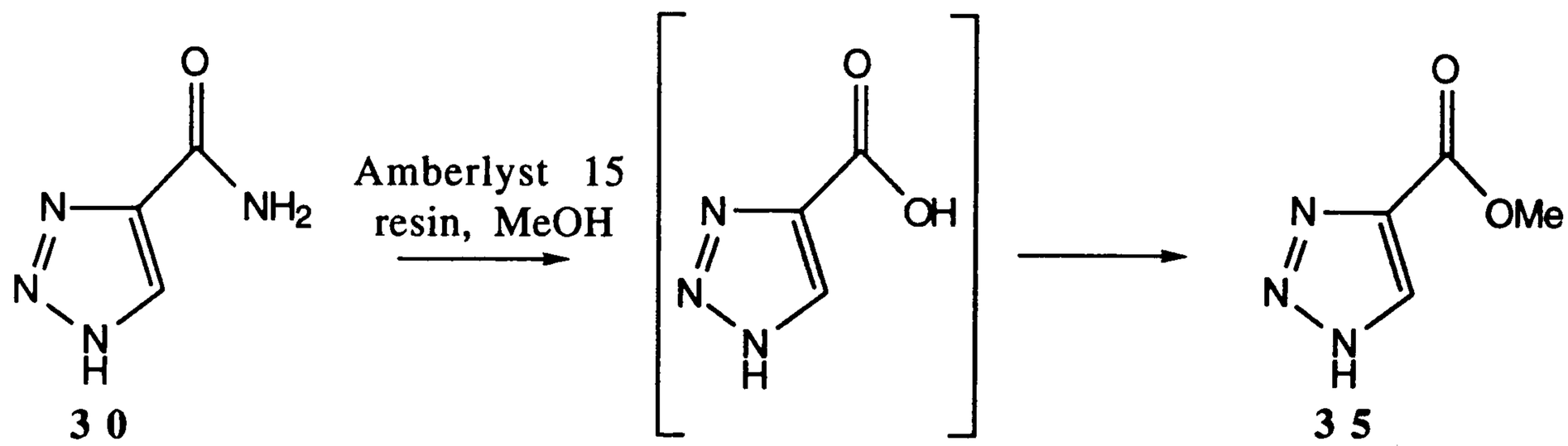
3 1 **3 2** **3 3** **3 4**

Scheme 2.6 Possible mechanism of the ring contraction of 5-diazouracil to 1,2,3-triazole-4-carboxamide

This reaction gave yields varying from 42% to 85% owing to the difficulties of maintaining a constant temperature in the autoclave. This is

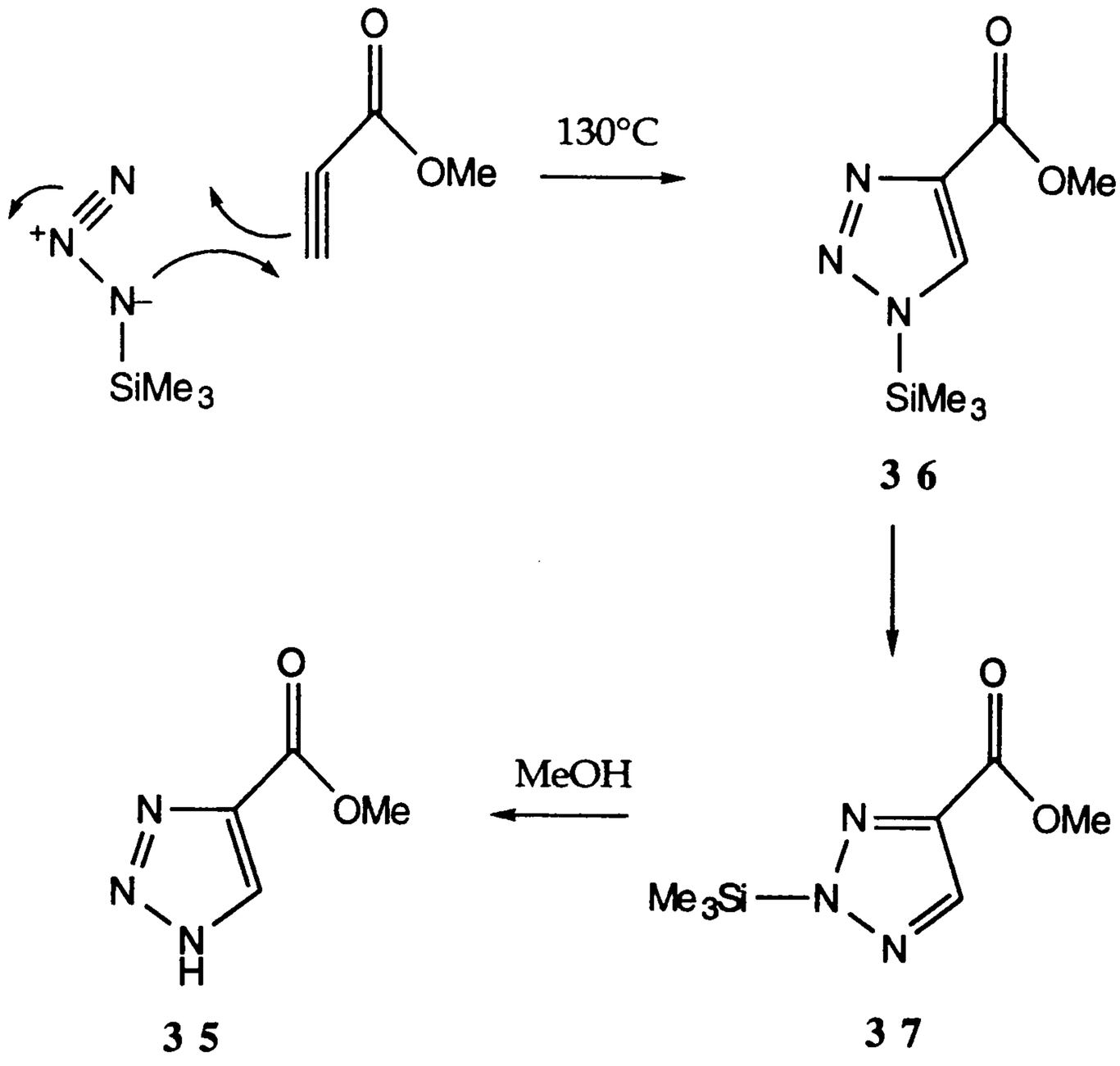
in contrast to the 98% yield .reported by Thurber *et al* for the same reaction.[184]

The methyl ester **35** of the carboxamide **30** was synthesised by gently refluxing the carboxamide and Amberlyst 15, a strong acid cation exchange resin, in methanol in a method adapted from Greenlee *et al* (Scheme 2.7).[188]



Scheme 2.7 Synthesis of methyl-1,2,3-triazole-4-carboxylate from the carboxamide[188]

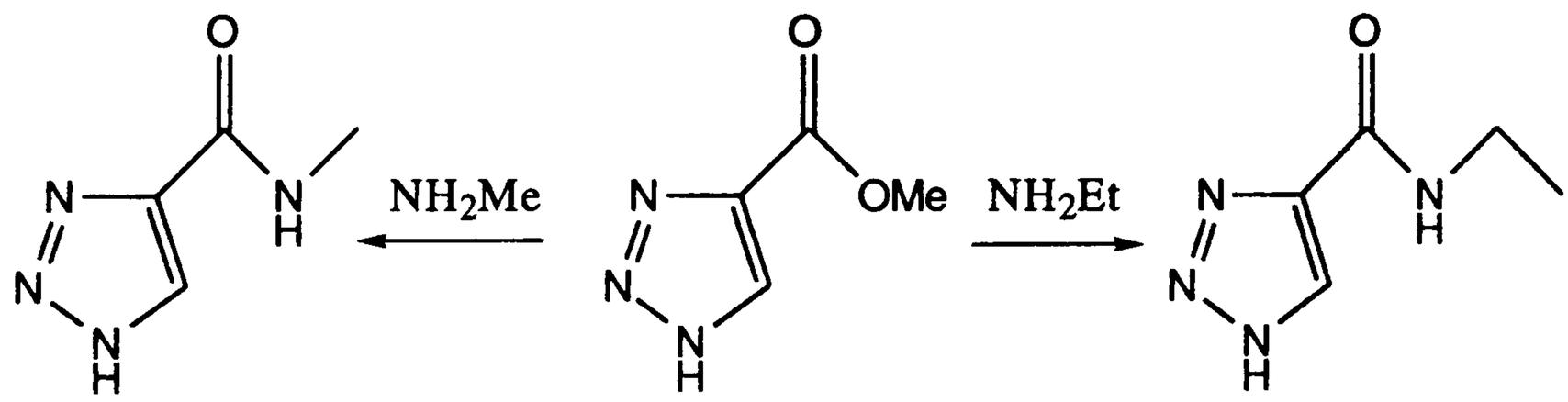
A more direct route to the same triazole ester **35** was the 1,3-dipolar cycloaddition of trimethylsilyl azide to methyl propiolate in an autoclave at 130°C.[189] Addition of methanol to the cooled reaction mixture brought about the smooth hydrolysis of the trimethylsilyl group, giving the methyl-1,2,3-triazole-4-carboxylate **35** in 83% yield (Scheme 2.8). The reaction is very regioselective and only the 4-substituted ester is formed. Compared to the direct addition of hydrazoic acid or sodium azide to alkynes, trimethylsilylazide provides a much safer route. The initial product is the 1-trimethylsilyl-methyl-1,2,3-triazole-4-carboxylate **36** but this rearranges to the 2-substituted isomer **37**[189] and is very labile to hydrolysis.



3 5 **3 6** **3 7**

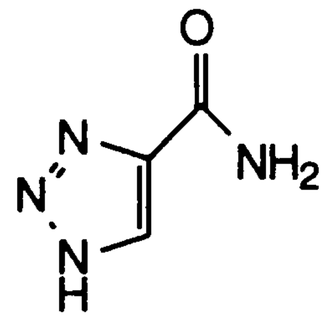
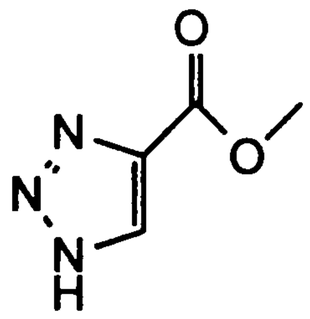
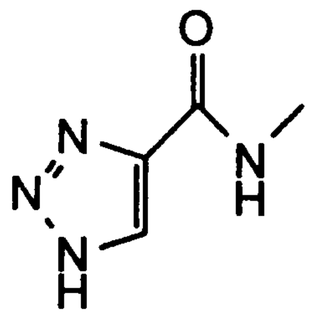
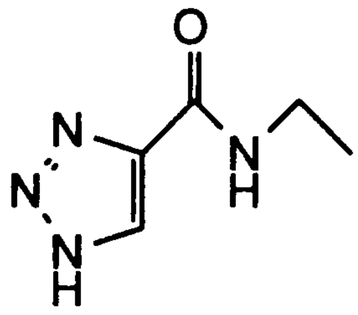
Scheme 2.8 Synthesis of methyl-1,2,3-triazole-4-carboxylate from an organic azide and acetylene[189]

The *N*-methyl- and *N*-ethyl-1,2,3-triazole-4-carboxamides **38** and **39** of the methyl ester **35** were synthesised by the same methods previously described for the 1,2,4-triazoles-3-carboxamides (Scheme 2.9).



3 8 **3 5** **3 9**

Scheme 2.9 Synthesis of 1,2,3-triazole-4-carboxamides

	Structure	% Yield
30		42-85
35		56 † 83 ‡
38		76
39		89

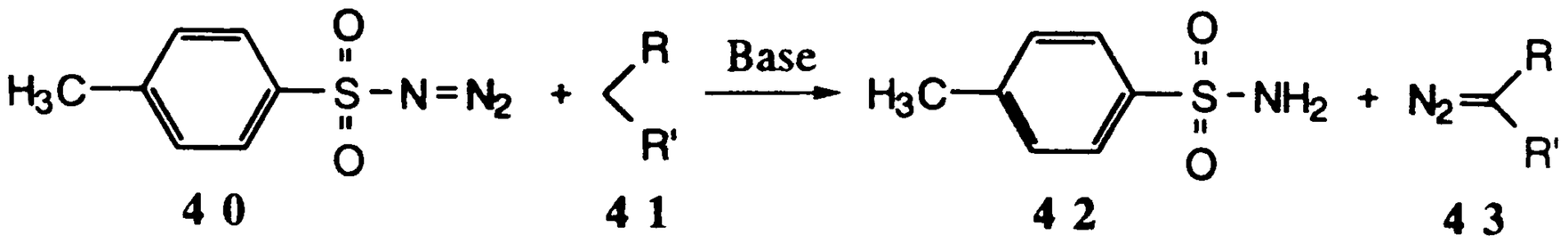
† Synthesised from 1,2,3-triazole-4-carboxamide[188] (Scheme 2.7)

‡ Synthesised from trimethylsilyl azide and methyl propiolate[189] (Scheme 2.8)

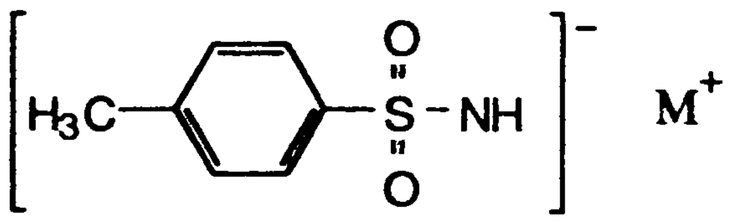
Table 2.2 4-Monosubstituted-1,2,3-triazoles synthesised

Synthesis of 1-*N*-Protected-4,5-disubstituted-1,2,3-triazole Bases

Sulfonyl azides **40** are used as a source of the diazo group where the dinitrogen is exchanged in a one-step reaction for two protons of an active methylene compound **41** in the presence of a base. The p-toluenesulfonamide **42** is generally separated from the newly formed diazo compound **43** as a salt **44** (Scheme 2.10).[190]



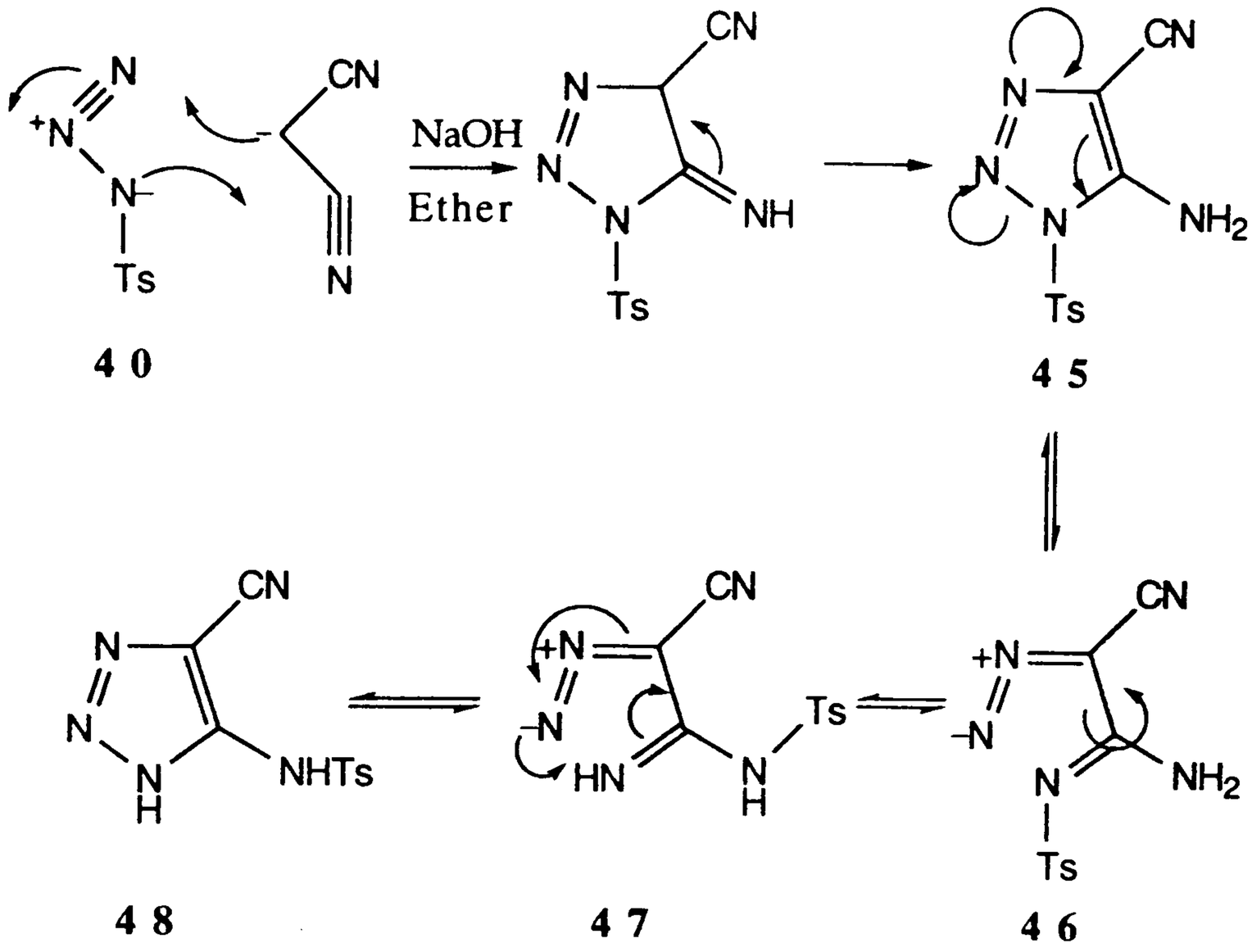
40 41 42 43



44

Scheme 2.10 Transfer of diazo groups from sulfonylazides

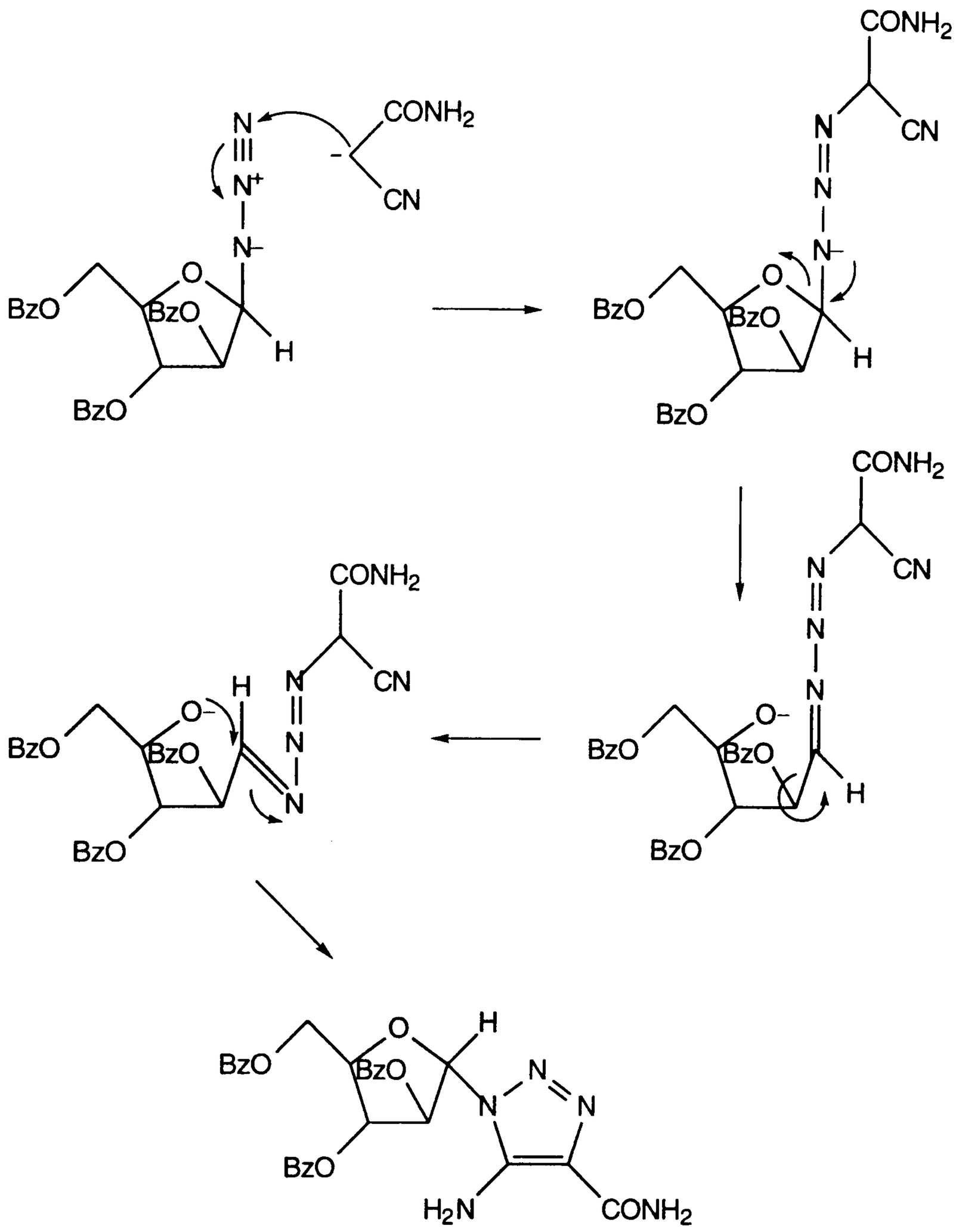
Reaction of the sulfonyl azide with malononitrile exhibits a novel reaction, where in aqueous sodium hydroxide or ethanol and potassium ethoxide a very stable 1:1 p-toluenesulfonylazide adduct is formed.[191] This adduct was eventually shown to be the 5-tosylamine-1-*H*-1,2,3-triazole-4-carbonitrile **48** (Scheme 2.11).[192] The 5-tosylamine **48** derivative is the more stable isomer formed in a Dimroth rearrangement of the initially formed intermediate, 5-amino-1-*N*-tosyl-1,2,3-triazole-4-carbonitrile **45**. The strongly electronwithdrawing effect of the tosyl group favours ring cleavage to form the diazo isomer **45-46** and the more nucleophilic amidine nitrogen, the one without the tosyl group, attacks the electron deficient terminal diazo nitrogen causing ring closure **47-48**. Though the reaction is reversible in pyridine[193] the facile rearrangement proved to be a problem and use of this sort of protecting group was abandoned.



40 45 48 47 46

Scheme 2.11 Synthesis of 5-amino-1-*N*-tosyl-1,2,3-triazole-4-carbonitrile followed by a Dimroth rearrangement

The base-catalysed condensation of the azides with activated methylene compounds is a well established route to 1-*H*-triazoles.[194] It is ideal for the regiospecific synthesis of 1,2,3-triazoles with an amino or hydroxy substituent at the 5 position and a carbonyl or aryl function at C-4. The stepwise mechanism of the reaction is best revealed by the anomerisation of the glycosyl azides on reaction with activated methylene compounds (Scheme 2.12).[59]



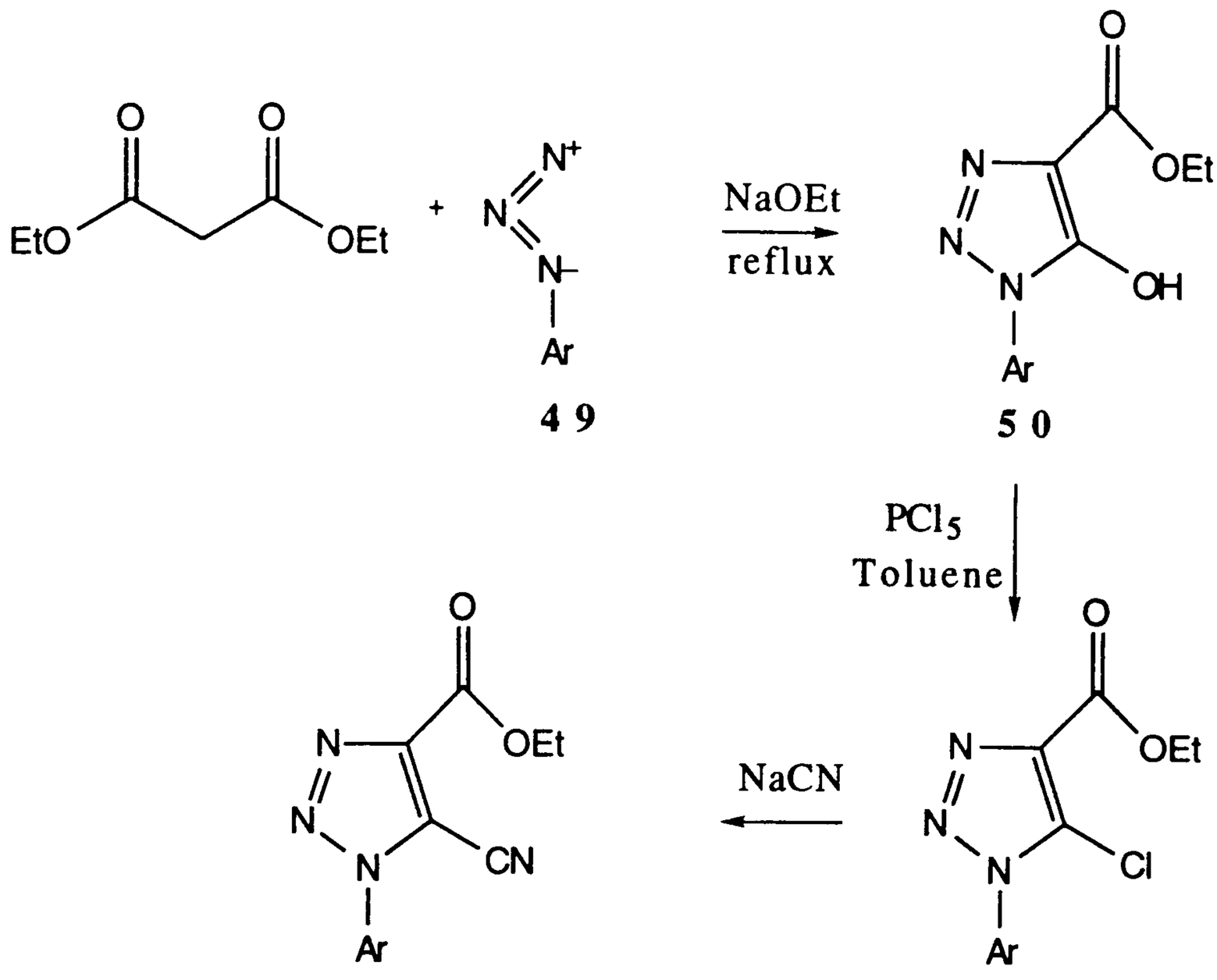
Scheme 2.12 Anomerisation of a glycosyl azide during a condensation reaction[59]

The proposed intermediates have been detected in solution or have been isolated from the solutions of reacting azides with aliphatic ketones.[195]

A versatile *N*-protecting group for the synthesis of 1-*N*-substituted-1,2,3-triazoles has been developed by Buckle *et al.*[196, 197] Protecting groups such as *N*-benzyl are too difficult to remove[198] and destroy the triazole

ring or substituents with the forcing conditions required. Others are too labile, as with the tosyl group[199] and trimethylsilyl group.[200] The 4-methoxybenzyl group, on the other hand, is stable under a wide range of experimental conditions but is sufficiently labile to be removed by solvolysis in trifluoroacetic acid in the presence of a variety of functional groups.[197]

The synthesis by Buckle *et al* incorporated the base-catalysed condensation with the 4-methoxybenzyl protecting group. The method was followed exactly as described to synthesise the 5-substituted-ethyl-1,2,3-triazole-4-carboxylates (Scheme 2.13).[197] The 4-methoxybenzylazide **49** was easily prepared from the corresponding 4-methoxybenzyl chloride and sodium azide. Condensation of 4-methoxybenzylazide **49** and diethylmalonate in the presence of a base gave the 5-hydroxyl-1-(4-methoxybenzyl)-ethyl-1,2,3-triazole-4-carboxylate **50** in moderate yields. Treatment of the hydroxyl triazole with phosphorous pentachloride in dry toluene gave the chloro derivative **51** and this in turn was displaced by the cyanide ion to give the carbonitrile triazole **52**. Each reaction gave yields of about 60%.

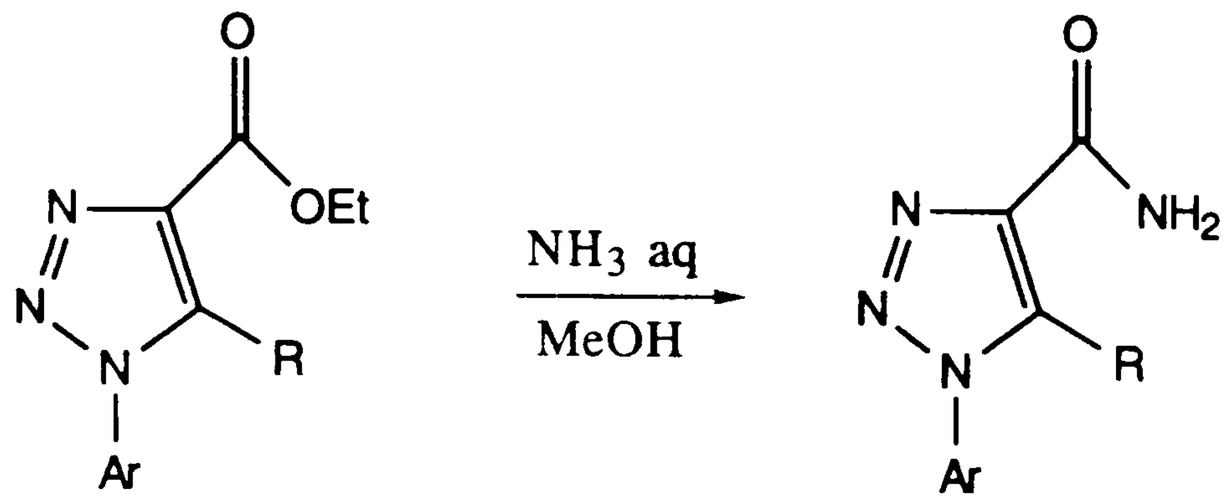


49 **50** **51** **52**

Ar = 4-$CH_3OC_6H_4CH_2$-

Scheme 2.13 Synthesis of *N*-protected-4,5-disubstituted-1,2,3-triazole bases[197]

Having followed the procedure of Thurber *et al* to its conclusion[197] the respective amides **53, 54** of the protected chloro **51** and cyano **52** derivatives were synthesised using methanolic ammonia or aqueous ammonia (Scheme 2.14).

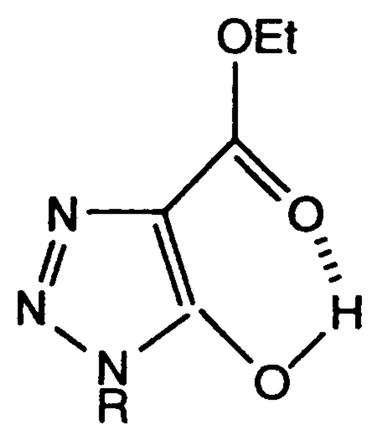


51, 52 **53, 54**

R = Cl, CN, Ar = 4-$CH_3OC_6H_4CH_2$-

Scheme 2.14 Synthesis of *N*-protected-5-substituted-1,2,3-triazole-4-carboxamide bases

The amide of the hydroxyl compound, protected or unprotected, could not be formed, possibly due to enolisation or hydrogen-bonding between the carbonyl group of the ester and the 5-hydroxyl group preventing nucleophilic attack (Fig. 2.3).

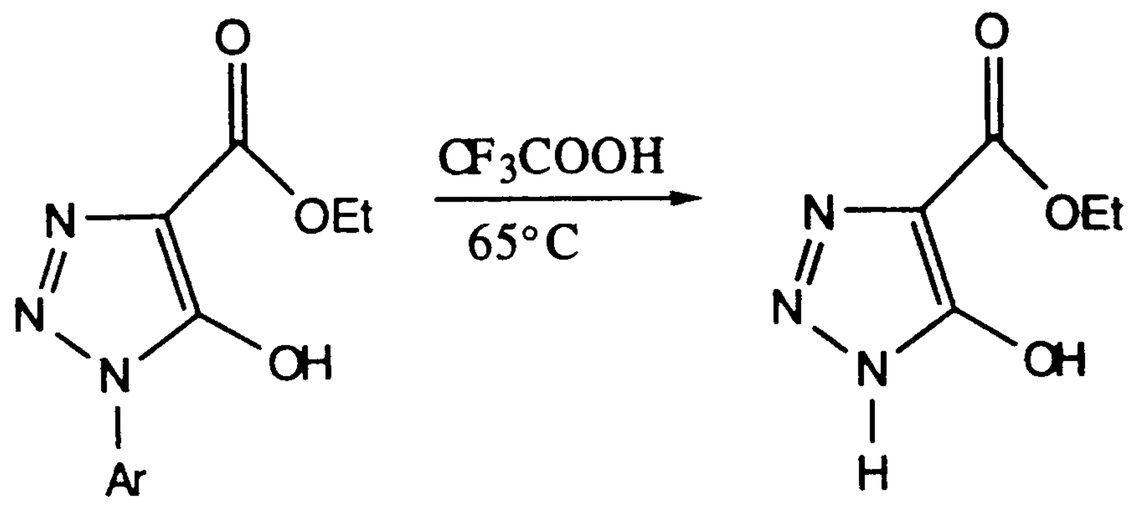


50 R = 4-$CH_3OC_6H_4CH_2$-

55 R = H

Fig. 2.3 Hydrogen bonding in ethyl-1,2,3-triazole-4-carboxylate preventing attack by ammonia

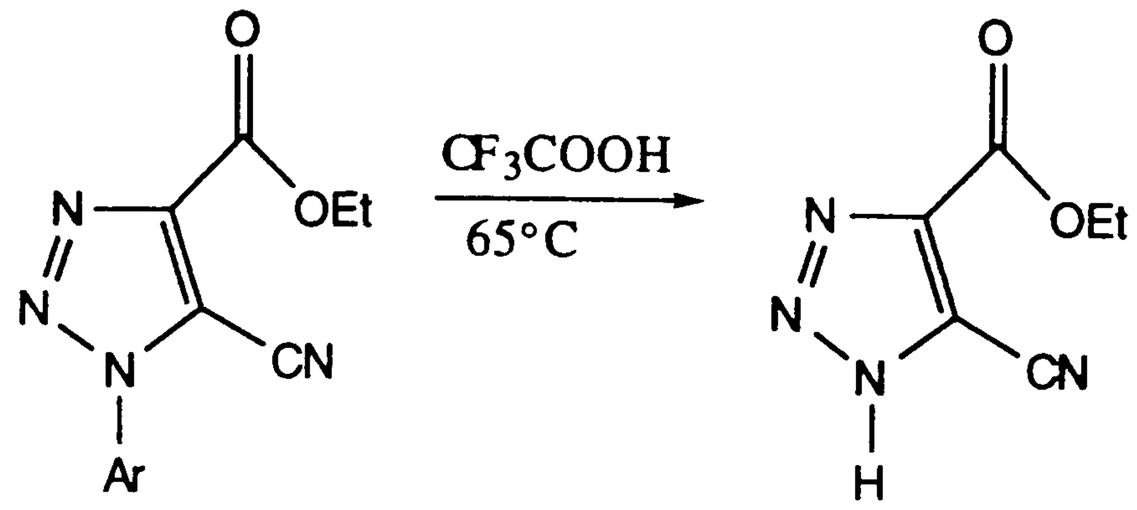
The 4-methoxybenzyl group was readily removed by solvolysis with trifluoroacetic acid at 65°C in yields ranging from 46% to 90% (Scheme 2.15, 2.16).



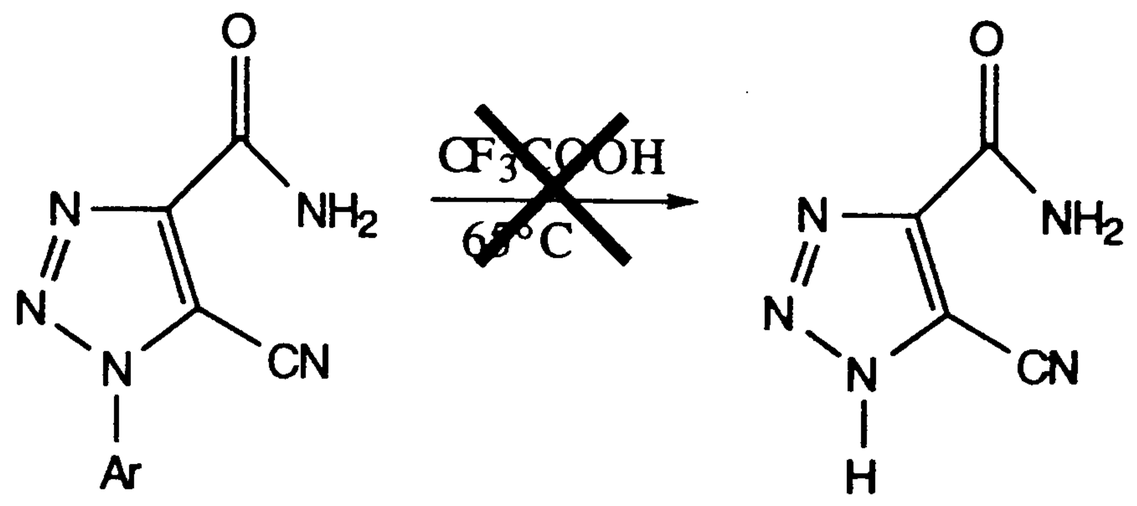
5 0 **5 5**

Ar = 4-$CH_3OC_6H_4CH_2$-

Scheme 2.15 *N*-Deprotection of the 5-hydroxyl-1,2,3-triazole base with trifluoroacetic acid



5 2 **56**

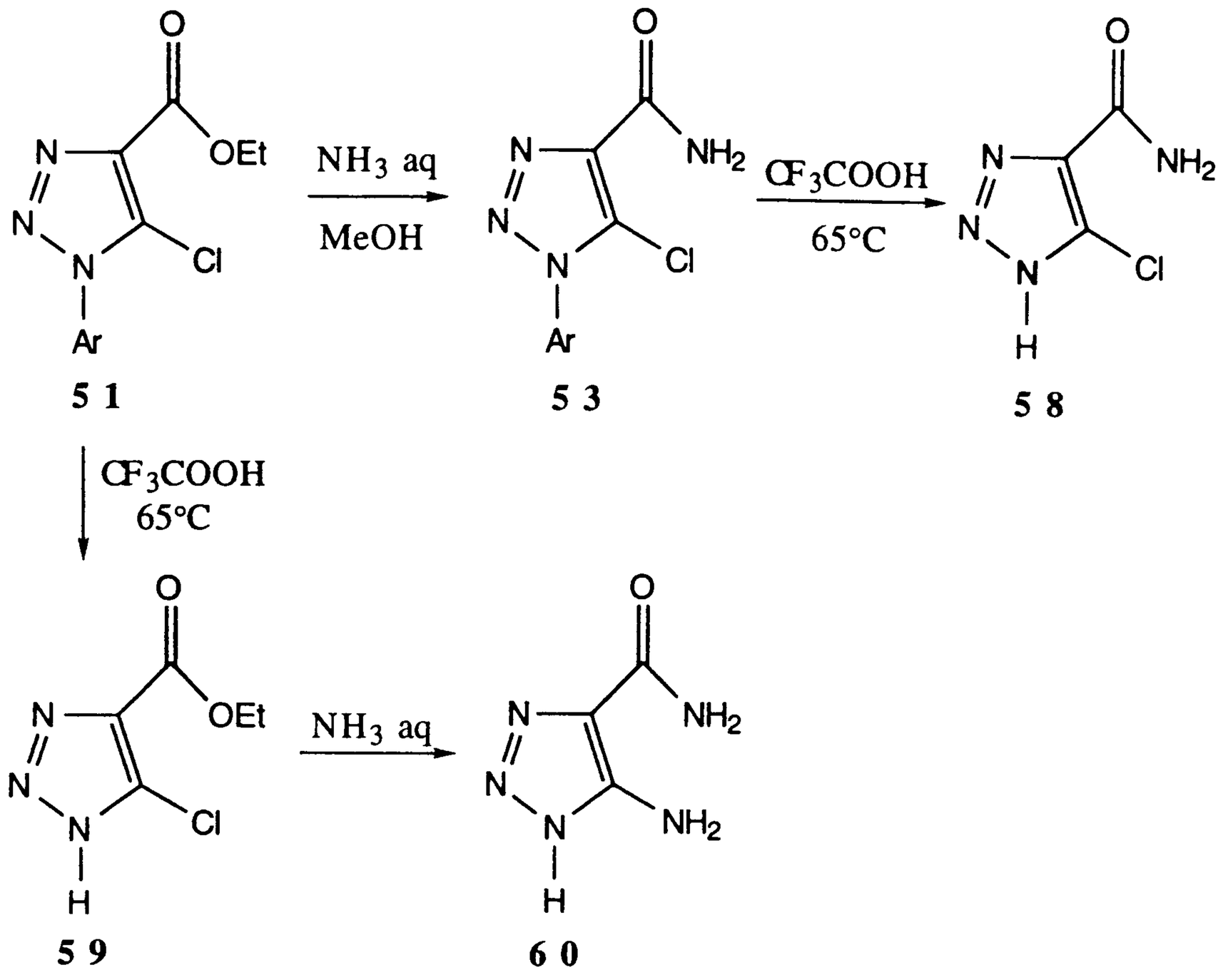


5 4 **5 7**

Ar = 4-$CH_3OC_6H_4CH_2$-

Scheme 2.16 *N*-Deprotection of the 5-cyano-1,2,3-triazole bases with trifluoroacetic acid

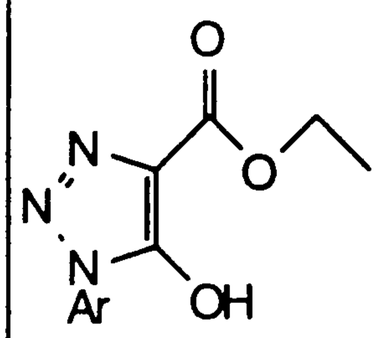
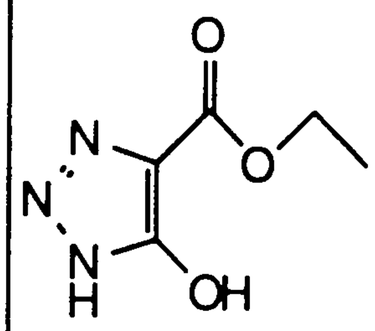
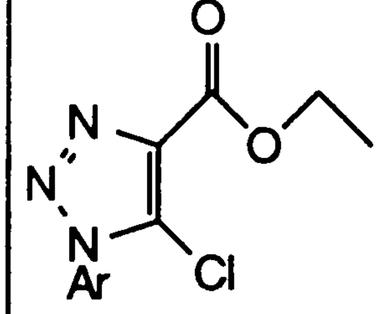
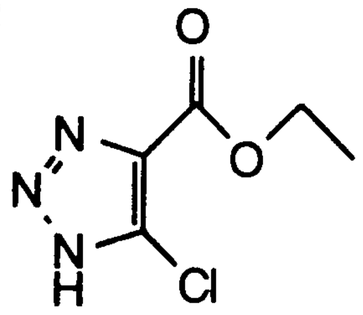
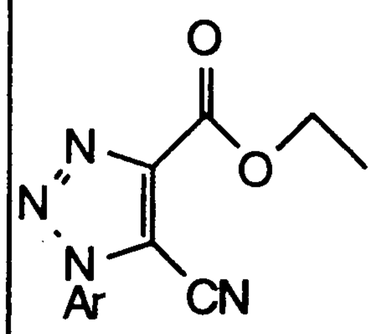
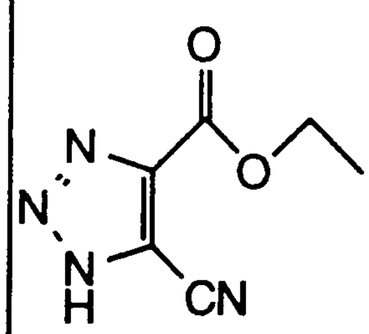
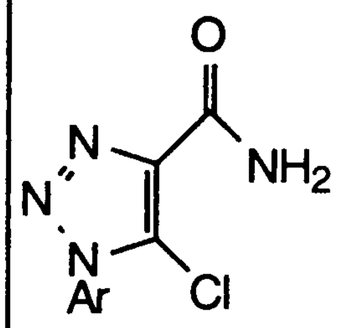
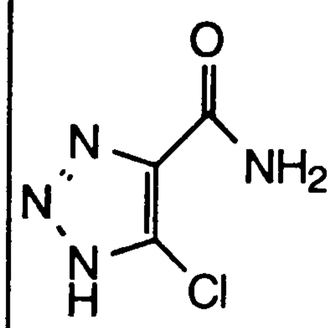
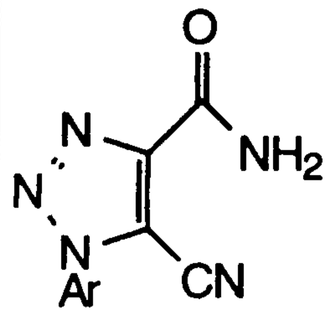
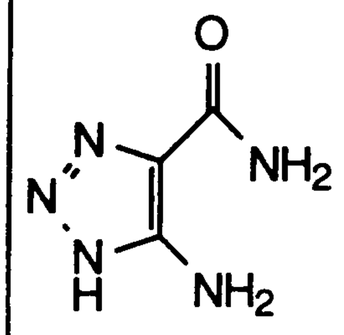
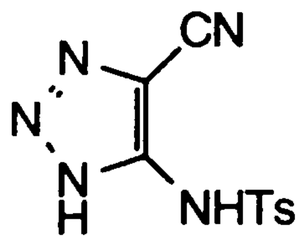
Neither the deprotection of the protected 5-cyano-1,2,3-triazole-4-carboxamide **54** nor the ammonolysis of ethyl 5-cyano-1,2,3-triazole-4-carboxylate **56** gave the required product **57** (Scheme 2.16). A gave the same result. Treatment of the unprotected ethyl-5-chloro-1,2,3-triazole-4-carboxylate **59** with methanolic ammonia gave the 5-amino-1,2,3-triazole-4-carboxamide **60**, whereas the protected chloro derivative **51** gave only one product, the protected chloro carboxamide **53** (Scheme 2.17).



Ar = 4-$CH_3OC_6H_4CH_2$-

Scheme 2.17 *N*-Deprotection of the 5-chloro-1,2,3-triazole bases with trifluoroacetic acid

This slow displacement of the 5-chloro group of methyl 1-benzyl-1,2,3-triazole-4-carboxylate by ammonia or methylamine has been previously reported. The only products isolated were the amides, leaving the chloro group untouched.[201]

	Structure	% Yield		Structure	% Yield
50		65	**55**		46
51		68	**59**		90
52		52	**56**		78
53		79	**58**		82
54		50	**60**		68
48		82			

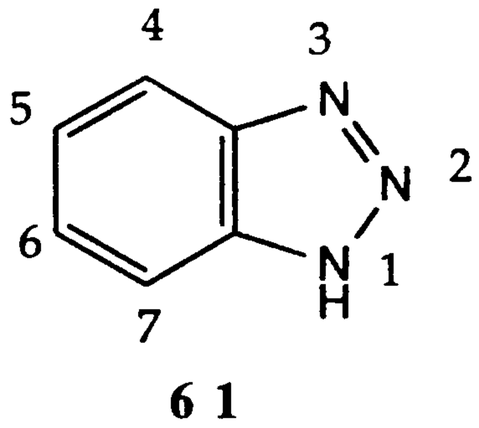
Ar = 4-$CH_3OC_6H_4CH_2$-, Ts = $CH_3C_6H_4SO_2$-

Table 2.3 4,5-Disubstituted-1,2,3-triazoles synthesised

Synthesis of Benzotriazole Bases

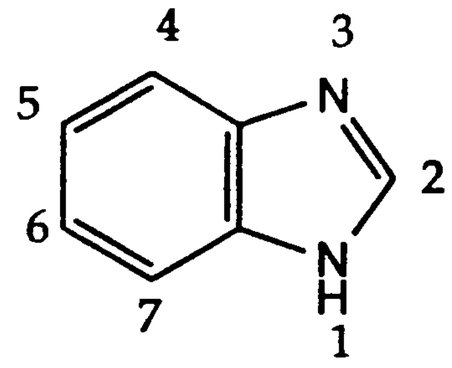
Little investigation has been conducted in the area of benzotriazole nucleosides. The first chemical synthesis of benzotriazole-2'-

deoxynucleosides was reported by Kazimierczuk *et al* in 1990.[202] Benzotriazole **61**[98] and benzimidazole **62**[111] can function successfully as substrates for *N*-deoxyribosyltransferases from *Lactobacillus leichmannii*, but the benzotriazole nucleoside was not isolated (Fig. 2.4).



6 1

Benzotriazole

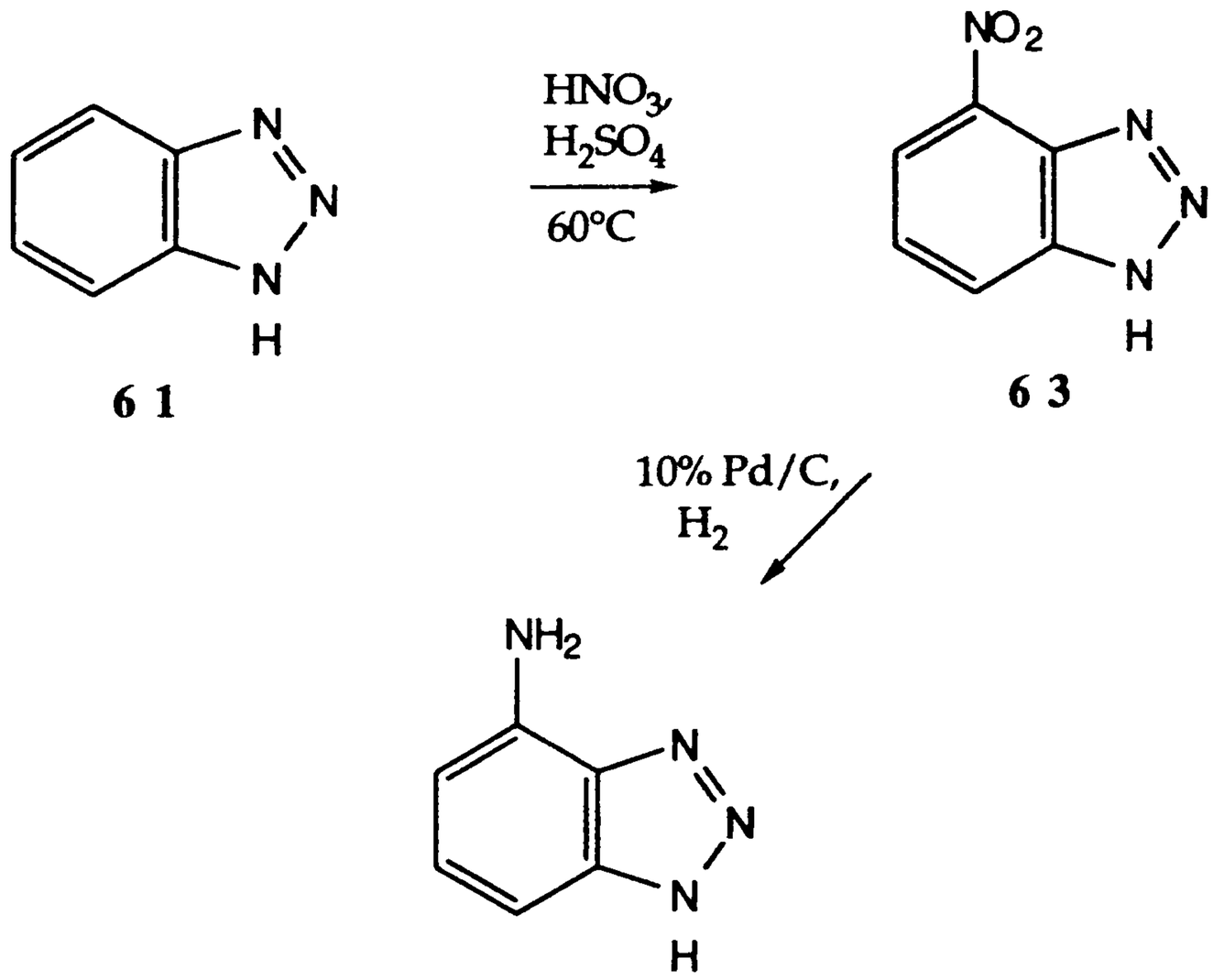


6 2

Benzimidazole

Fig. 2.4 Acceptor bases for *N*-deoxyribosyltransferases from *Lactobacillus leichmannii*

4-Nitrobenzotriazole **63**[203] and 4-aminobenzotriazole **64**[202] were synthesised as described by standard methods in 84% and 70% yields, respectively (Scheme 2.18).



6 1 **6 3** **6 4**

Scheme 2.18 Synthesis of 4-substituted benzotriazole bases

The parent compound **61** was the only one found to act as an acceptor for the *N*-deoxyribosyltransferase, as will be dicussed in Chapter Three so the synthesis of other benzotriazoles was not pursued.

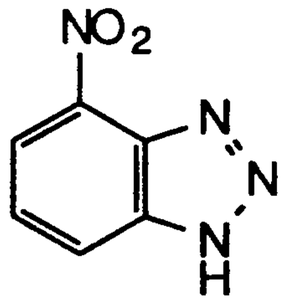
	Structure	% Yield
63		84
64		70

Table 2.4 4-Substituted benzotriazole bases synthesised

The results of the bases synthesised as acceptors for the reaction catalysed by the *N*-deoxyribosyltransferases from *Lactobacillus leichmannii* will be discussed in Chapter Three.

The results of Chapter Two are summarised in the following Tables 2.1 and 2.5

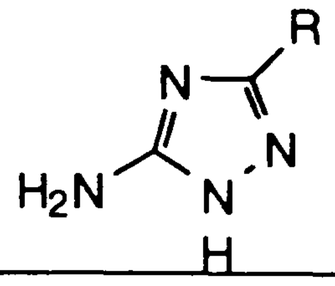
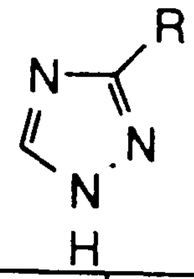
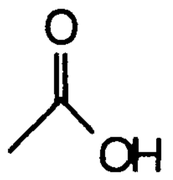
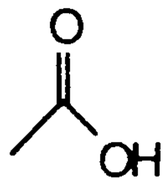
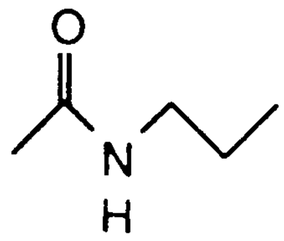
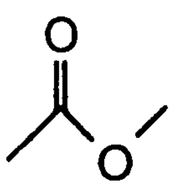
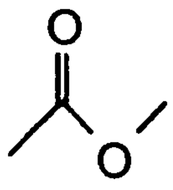
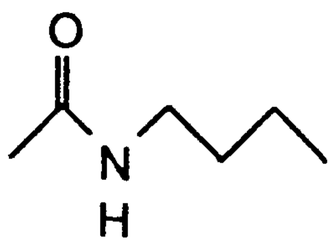
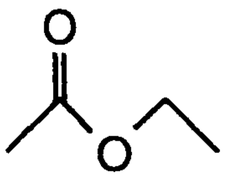
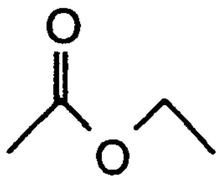
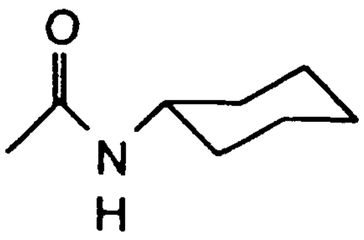
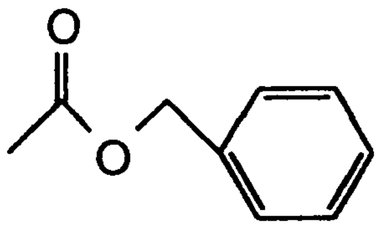
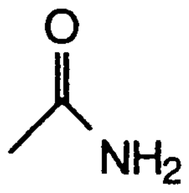
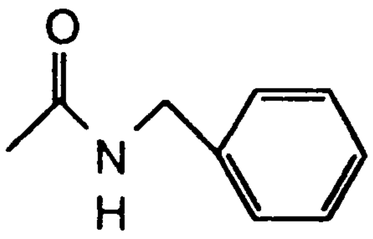
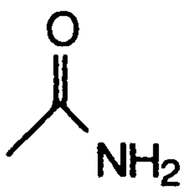
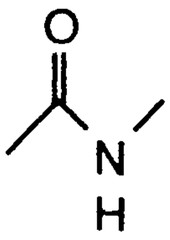
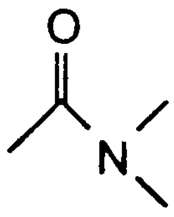
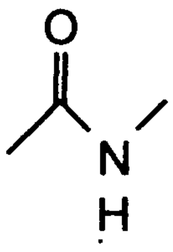
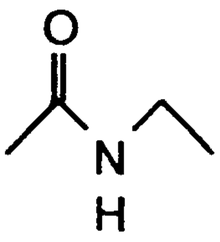
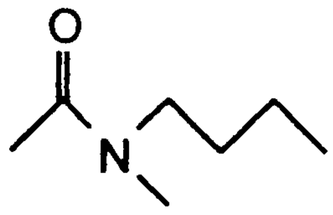
									
	R=	Yield %		R=	Yield %		R=		Yield %
10		98	**15**		94	**21**			72
11		78	**16**		89	**22**			79
12		53	**17**		50	**23**			37
27		15	**18**		94	**24**			25
13		90	**19**		81	**25**			68
14		97	**20**		92	**26**			40

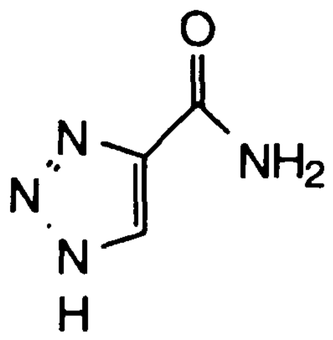
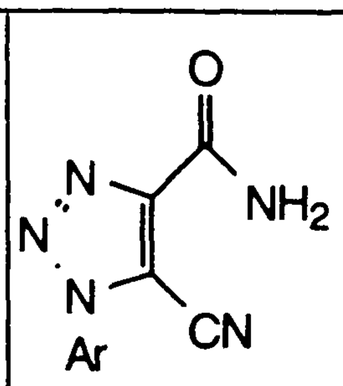
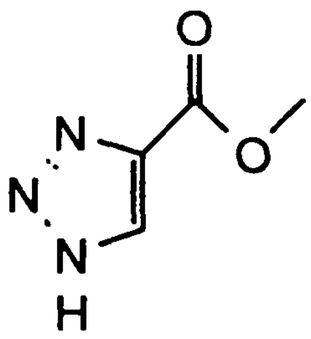
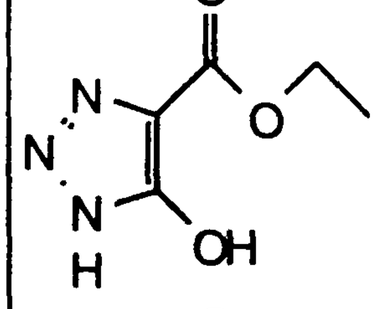
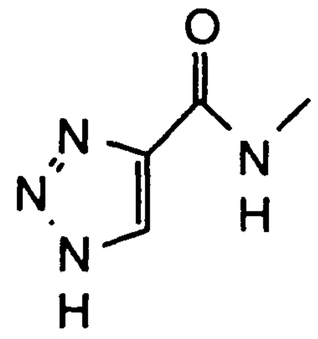
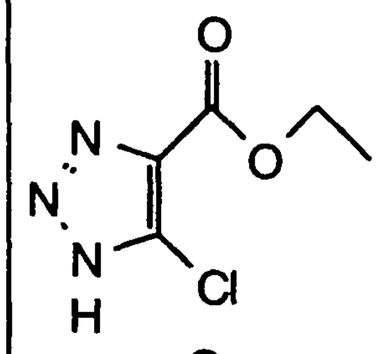
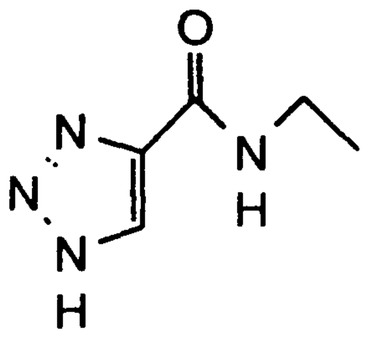
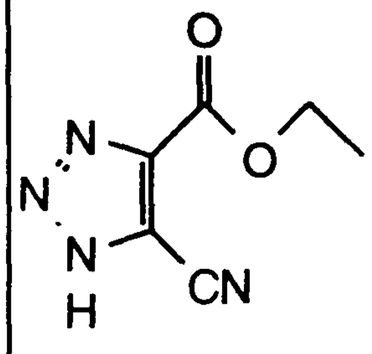
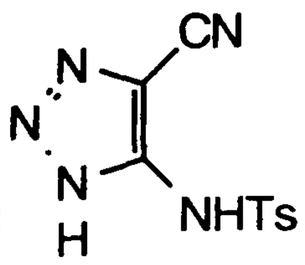
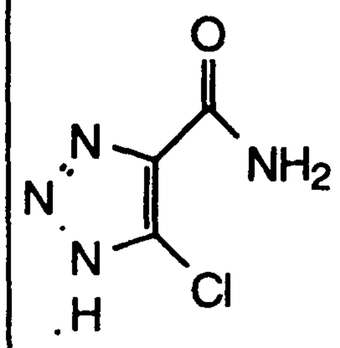
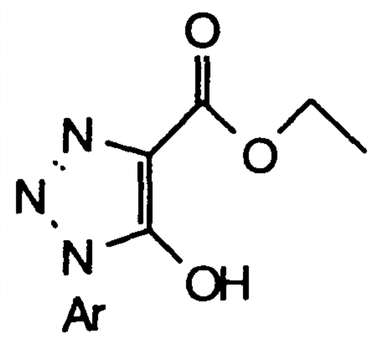
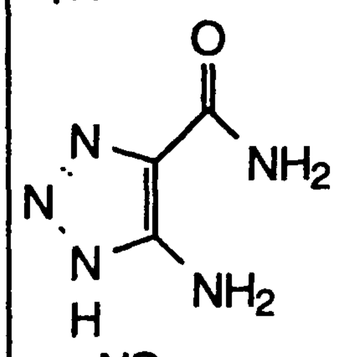
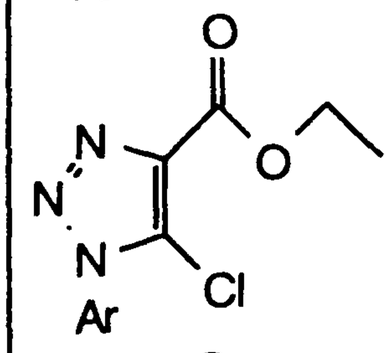
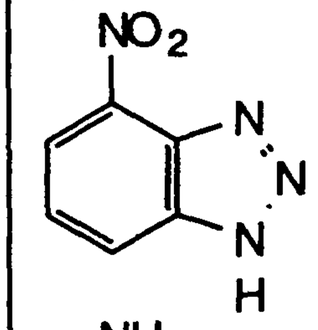
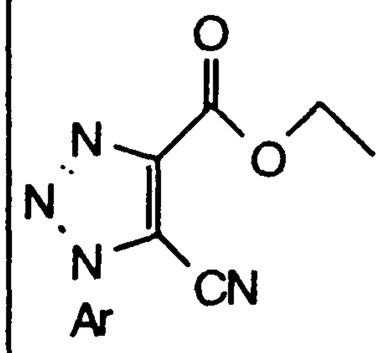
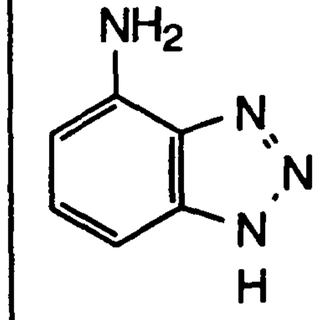
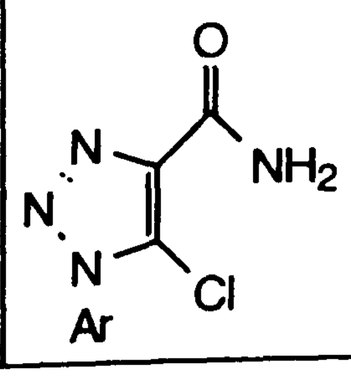
Table 2.1 Summary of 1,2,4-triazoles synthesised

Table 2.5 Summary of 1,2,3-triazoles synthesised

Ar = 4-$CH_3OC_6H_4CH_2$-, Ts = $CH_3C_6H_4SO_2$-

† Synthesised from 1,2,3-triazole-4-carboxamide[188] (Scheme 2.7)

‡ Synthesised from trimethylsilyl azide and methyl propiolate[189] (Scheme 2.8)

Structure	% Yield	Structure	% Yield
30 	42-85	54 	50
35 	56 † 83 ‡	55 	46
38 	76	59 	90
39 	89	56 	78
48 	82	58 	82
50 	65	60 	68
51 	68	63 	84
52 	52	64 	70
53 	79		

CHAPTER 3

ENZYMATIC SYNTHESIS OF NUCLEOSIDES

Background

Synthesis of Triazole Nucleoside Analogues

There are numerous routes to the synthesis of triazole nucleosides by either modification of an existing nucleoside, fusion of a sugar and base to form the nucleoside or the *de novo* synthesis, building the base onto the sugar. As discussed in Chapter One, these procedures have difficulties and much interest has been directed towards the development of enzymatic methods for the synthesis of nucleoside analogues where precise stereochemical and regiochemical control is possible without the use of protecting groups on reactive residues. *N*-Deoxyribosyltransferases are specific and glycosylate only one nitrogen in a heterocyclic base to give a nucleoside with a β–configuration. Several enzymatic syntheses of nucleoside analogues have been described[65, 108-111, 114] using a crude nucleoside *N*-deoxyribosyltransferase from *Lactobacillus leichmannii*. This route is complementary to one involving a combination of purine and pyrimidine phosphorylases. The availability of enzymatic synthetic methods means that a range of novel nucleosides is now accessible.

N-Deoxyribosyltransferases from *Lactobacillus leichmannii*

In this chapter, exploration of the use of *N*-deoxyribosyltransferases specifically from *Lactobacillus leichmannii* will be discussed in more detail. *Lactobacillus leichmannii* is a member of the subdivision of

obligately homofermentative lactobacilli which ferment glucose almost entirely to lactic acid. These lactic acid bacteria occur as regular, non-sporing, gram-positive rods with rounded ends (0.5-0.8 x 2-9 mm). The cells of *Lactobacillus leichmannii* occur singly, in pairs and in short chains, and can be isolated from milk, cheese, compressed yeast and grain mash. They require not only carbohydrates as energy and carbon sources, but nucleotides, amino acids and vitamins which are essential for growth.[204] Both *N*-deoxyribosyltransferase I and *N*-deoxyribosyltransferase II have been purified from *Lactobacillus leichmannii*,[98] but for the synthetic reactions a crude preparation of the enzyme is employed.

It has been shown that a wide range of bases act as competent acceptors with *N*-deoxyribosyltransferases but only very minor modifications on the sugar moiety are tolerated (Fig. 3.1).[88, 98, 107, 110] Analogues that lack an hydroxyl group in the 3'- or 5'-position are accepted by the enzyme as glycosyl donors.[93, 114, 205] However, transfer of the 2,3-dideoxyribosyl moiety occurs at a much slower rate than the natural 2-deoxyribosyl substrate.[112, 206]

R = H, OH R = H, OH, F, Cl, I

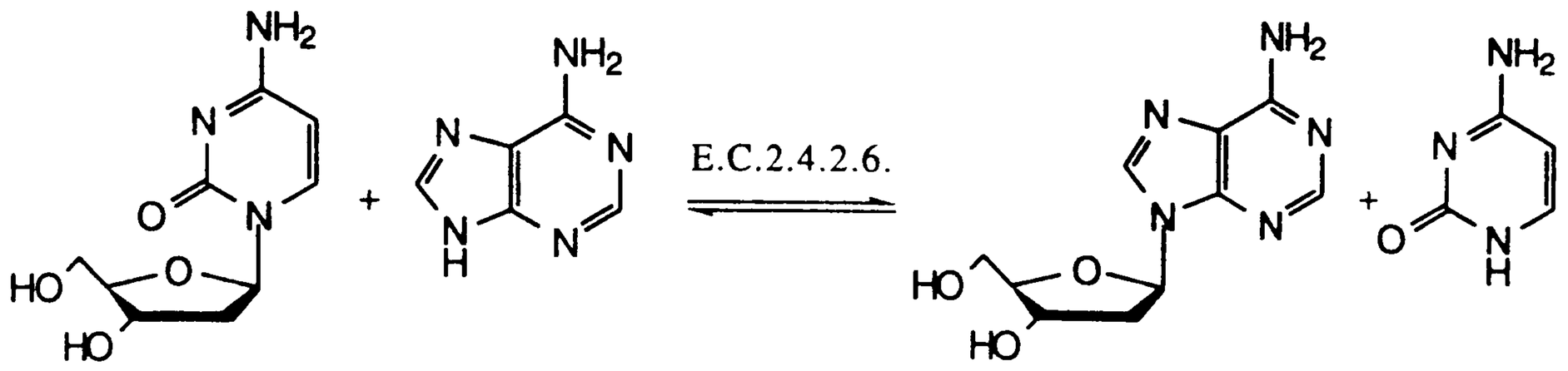
Fig. 3.1 Sugar moieties accepted as glycosyl donors by *N*-deoxyribosyltransferase

Enzymatic Synthesis of Nucleoside Analogues

Purification of Nucleoside *N*-Deoxyribosyltransferases (E.C.2.4.2.6) from *Lactobacillus leichmannii*

Bacteria are one of the most favoured sources of enzymes and the purification of a crude preparation of *N*-deoxyribosyltransferases from lactic acid bacteria was fairly simple. *Lactobacillus leichmannii* was grown for 24 hours at 37°C. The cells were then harvested by centrifugation during their stationary phase, when the highest levels of *N*-deoxyribosyltransferases are present. The cells were broken open by the use of a French pressure cell, which forces the cell suspension through a restricted orifice at high pressure. After cell breakage, the cell debris was removed by centrifugation. The resulting yellow liquid was dialysed to remove salts and thus yielded the crude preparation of enzyme. This preparation contained a multitude of different enzymes which could interfere with the transfer reactions. To remove the need for any further purification steps a technique, whereby the other enzymes, such as hydrolases and deaminases, in the crude preparation are inhibited by the addition of 10% (v/v) ethylene glycol, has been developed.[109] This protects the products of the reaction from degradation by other enzymes present in the crude preparation, improving yields and removing the necessity of purifying the enzyme, greatly simplifying the procedure. This crude enzyme preparation was then assayed for its protein concentration using the dye-binding BioRad protein assay and assayed to determine the number of units present.

The enzymatic transfer reaction involves the exchange of the base moiety in the nucleoside with a free base molecule to yield a new nucleoside (Scheme 3.1).



dC A dA C

Scheme 3.1 Standard enzymatic transfer reaction

The standard assay system used to calculate the number of units present is as shown in Scheme 3.1: 2'-deoxycytidine (dC) as the glycosyl donor while adenine (A) acts as the glycosyl acceptor. The rate at which the new nucleoside, 2'-deoxyadenosine (dA), was produced was followed by reverse-phase HPLC, detected by UV at 254 nm. The units and specific activity of a sample of enzyme will vary with the assay system used. Therefore, a unit of enzyme must be defined for the particular assay system used, as can be found in General Materials and Methods (Chapter Five).

The transfer reaction is reversible. Accordingly, if it is to have any use as a synthetic tool it must be displaced as fully as possible in the direction of the products. The adaptations which are generally used to push the equilibrium to the right-hand-side are:

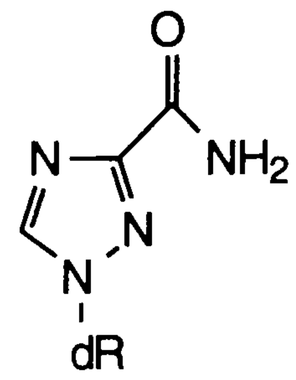
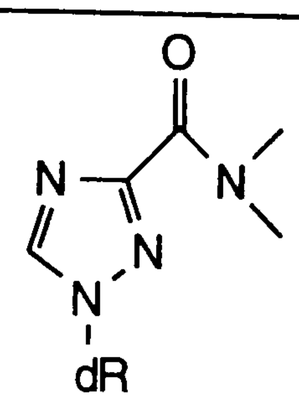
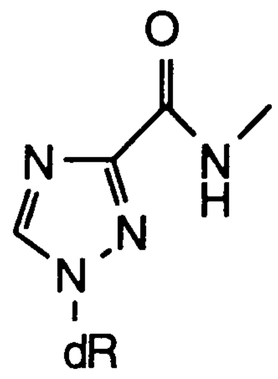
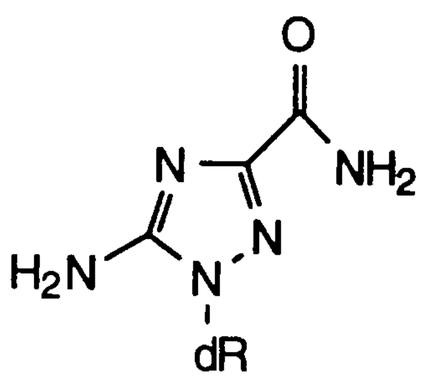
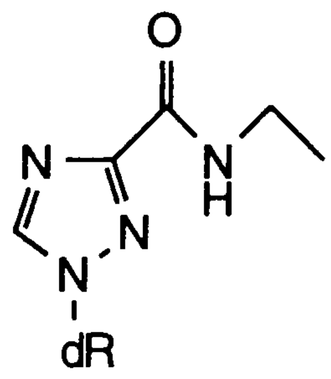
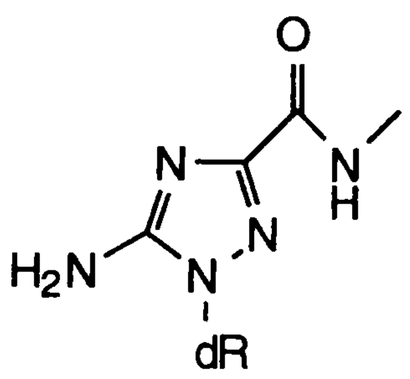
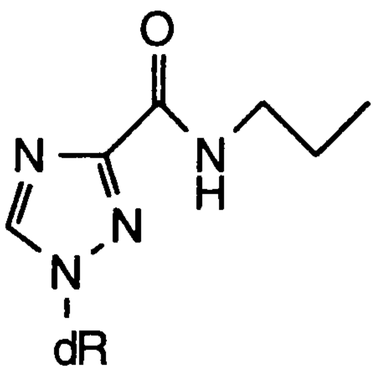
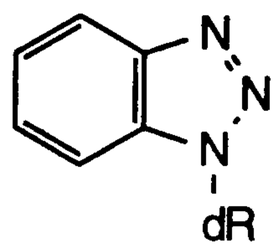
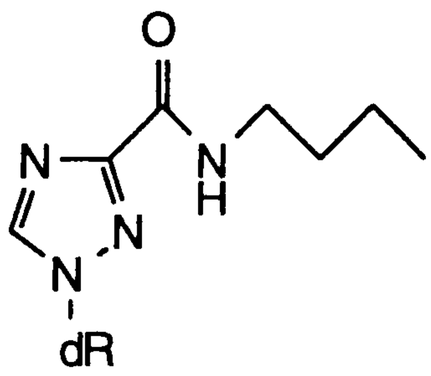
- a ratio of 3:1 donor nucleoside to acceptor base;
- a donor nucleoside which has a high affinity for the enzyme;
- a donor nucleoside in which the base is a good leaving group.

For the synthesis of 2'-deoxynucleosides, a glycosyl donor is required which will give a high levels of transfer with a large number of acceptor bases. From substrate specificity studies performed it has been shown that

2'-deoxycytidine is a better 2-deoxyribosyl donor than thymidine, 2'-deoxyuridine or any of the other naturally occuring nucleosides.[88, 206]

Synthesis of Triazole Nucleoside Analogues

Using a crude preparation of *N*-deoxyribosyltransferase from *Lactobacillus leichmannii* the transfer of 2-deoxyribose from 2'-deoxycytidine or thymidine to the triazole bases in the presence of ethylene glycol was followed on an analytical scale. The progress of the reaction was monitored by reverse phase HPLC detected by UV at 214 nm. Transfer reactions were followed until equilibrium was attained or, in the case of unsuccessful reactions, until complete hydrolysis of the donor nucleoside occurred. Table 3.1 shows the nucleosides made using the *N*-deoxyribosyltransferase catalysed reactions. When transfer occurred, only one new compound was detected by HPLC.

	Nucleoside	% Conv.	% Yield		Nucleoside	% Conv.	% Yield
65		62	35	**70**		50	43
66		62	24	**71**		75	64
67		95	89	**72**		66	43
68		54	29	**73**		60	50
69		73	42				

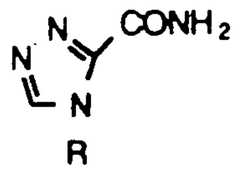
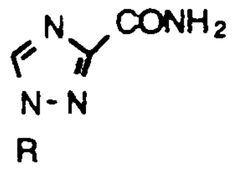
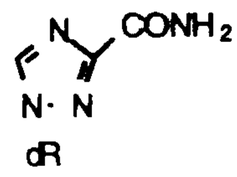
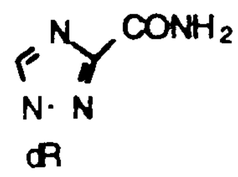
dR = 2-deoxyribose

Table 3.1 Triazole nucleosides synthesised

Discrepancies between the percentage conversion and the final yield are explained by the difficulties encountered in the work-up procedure. Similarities in the Rfs of some reactants and products required final purification by semipreparative HPLC, after incomplete separation by flash chromatography. In most cases the percentage conversion was

higher in the analytical than in the preparative *N*-deoxyribosyltransferase reaction. The difficulty of removing ethylene glycol at the end of the reaction made it necessary to reduce the overall amount used. The consequent increase in the concentration of the substrates and products, in comparison to that used on the analytical scale, led to problems of solubility and appeared to decrease the reaction rate.

The enzymatic glycosylation of triazoles can, in theory, lead to three products depending on the position of glycosylation. However, only one product was produced. A combination of ^{1}H and ^{13}C NMR together with nuclear Overhauser enhancement (nOe) experiments was used to determine the site of attachment of the sugar residue in 1,2,3-triazole and 1,2,4-triazole nucleosides. The site of glycosylation at N-1 agrees with ^{1}H and ^{13}C chemical shift NMR studies on 1-β-D-ribofuranosyl-1,2,4-triazoles.[207] The data suggests, allowing for the difference in machines and solvents, that the deoxynucleosides synthesised are glycosylated at N-1 and not N-4 of the 1,2,4-triazole base (Table. 3.2). Thus N-1 is the site of attachment of all the triazole nucleosides synthesised.

Compound		^{13}C NMR			^{1}H NMR
		C-3	C-5	C=O	H-5
75*		151.2	148.5	159.7	8.16
7*		158.1	146.0	161.5	8.91
65		157.19	144.83	160.60	8.81
65		158.1	146.1	163.4	8.74

R = ribose

dR = 2-deoxyribose

* Figures from Kreishman *et al*[207] in DMSO-d^6

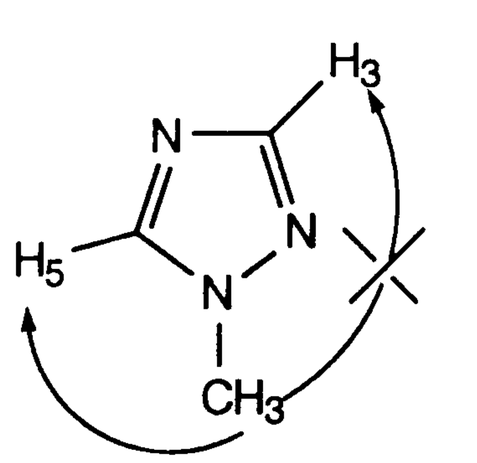
Figures from Sanghvi *et al*[135] in DMSO-d^6

In MeOH-d^4

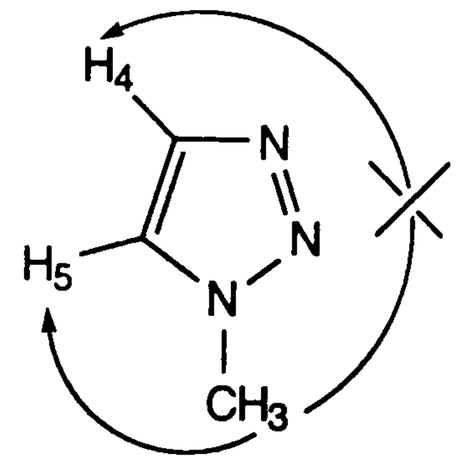
Table 3.2 Comparison of ^{13}C and ^{1}H NMR data to confirm N-1 glycosylation of the triazole nucleoside

Nuclear Overhauser enhancement (nOe), whereby a chosen signal is irradiated and the percentage enhancement of the other signals in the spectrum are observed, utilises the through-space interaction between protons. Holzer has developed a method for the assignment of triazole proton resonances in 1-substituted 1-*H*-1,2,3-triazoles[208] and 1-substituted 1-*H*-1,2,4-triazoles.[209] Upon the irradiation of the proton on the *N*-1-substituent of the 1,2,4-triazole **76** a marked enhancement of the H-5 was observed but the H-3 remained nearly unaffected. The same was found to be true with the 1,2,3-triazoles **77** (Fig. 3.2). Studies on a series of 1-

substituted triazoles confirmed this to be a general and unambiguous method of assignment.



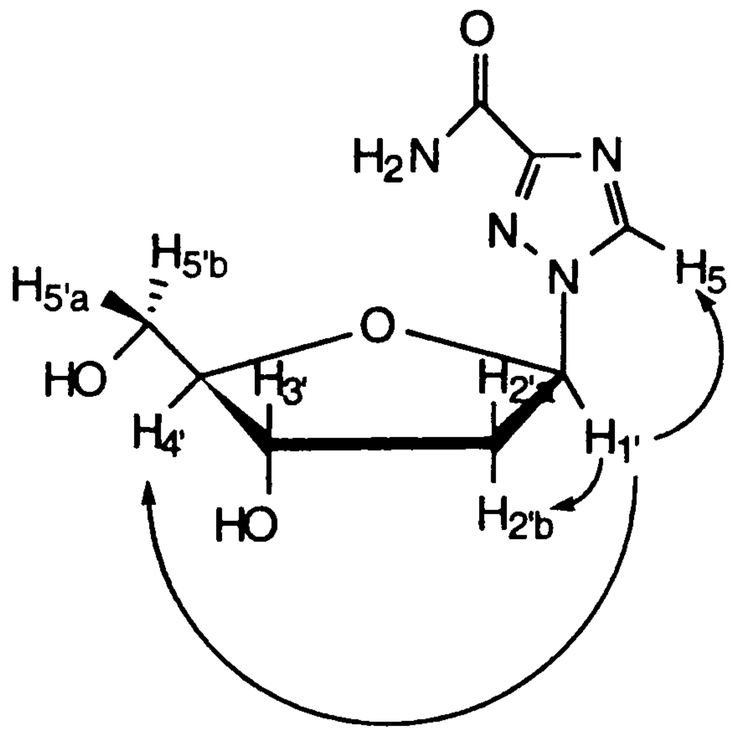
76



77

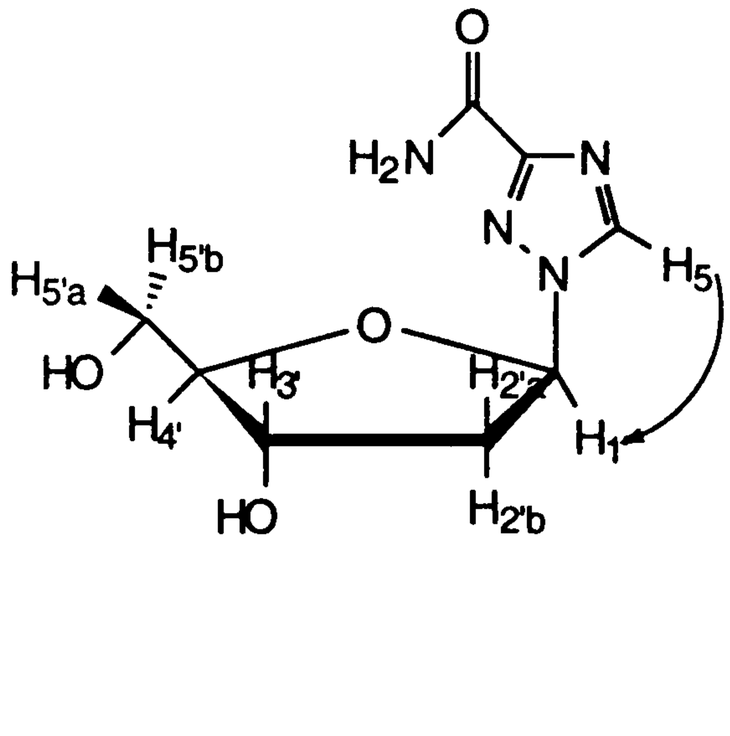
Fig. 3.2 Sites of nuclear Overhauser enhancements in the ^{1}H NMR spectrum of 1,2,4-triazoles and 1,2,3-triazoles

In the area of nucleosides, the anomeric configuration has been assigned by the use of nOe spectroscopy.[210, 211] It is easy to distinguish the α– and β–anomers by saturating H-1' and measuring the nOe enhancement at H-3' and H-4'. Enhancement at H-4' proves the β–configuration at the anomeric centre **78** while α– anomers show an nOe at H-3' upon irradiation of H-1'.



Irradiation at H-1'

78



Irradiation at H-5

79

Fig. 3.3 Sites of nuclear Overhauser enhancements in the ^{1}H NMR spectrum of 2'-deoxyribavirin

In all but one case, irradiation at H-1' gave enhancement of the signal due to H-4' and H-2'b **78** (Fig. 3.3). Therefore, H-1', H-2'b and H-4' must be on the same side of the ring, that is the underside, confirming the β-orientation of the glycosidic linkage. The *syn* and *anti* conformations of nucleosides in solution have been analysed by the same method.[212, 213] Irradiation of the signal due to H-5 of the triazole ring only showed enhancement of the signal at H-1' **79** (Fig. 3.3).

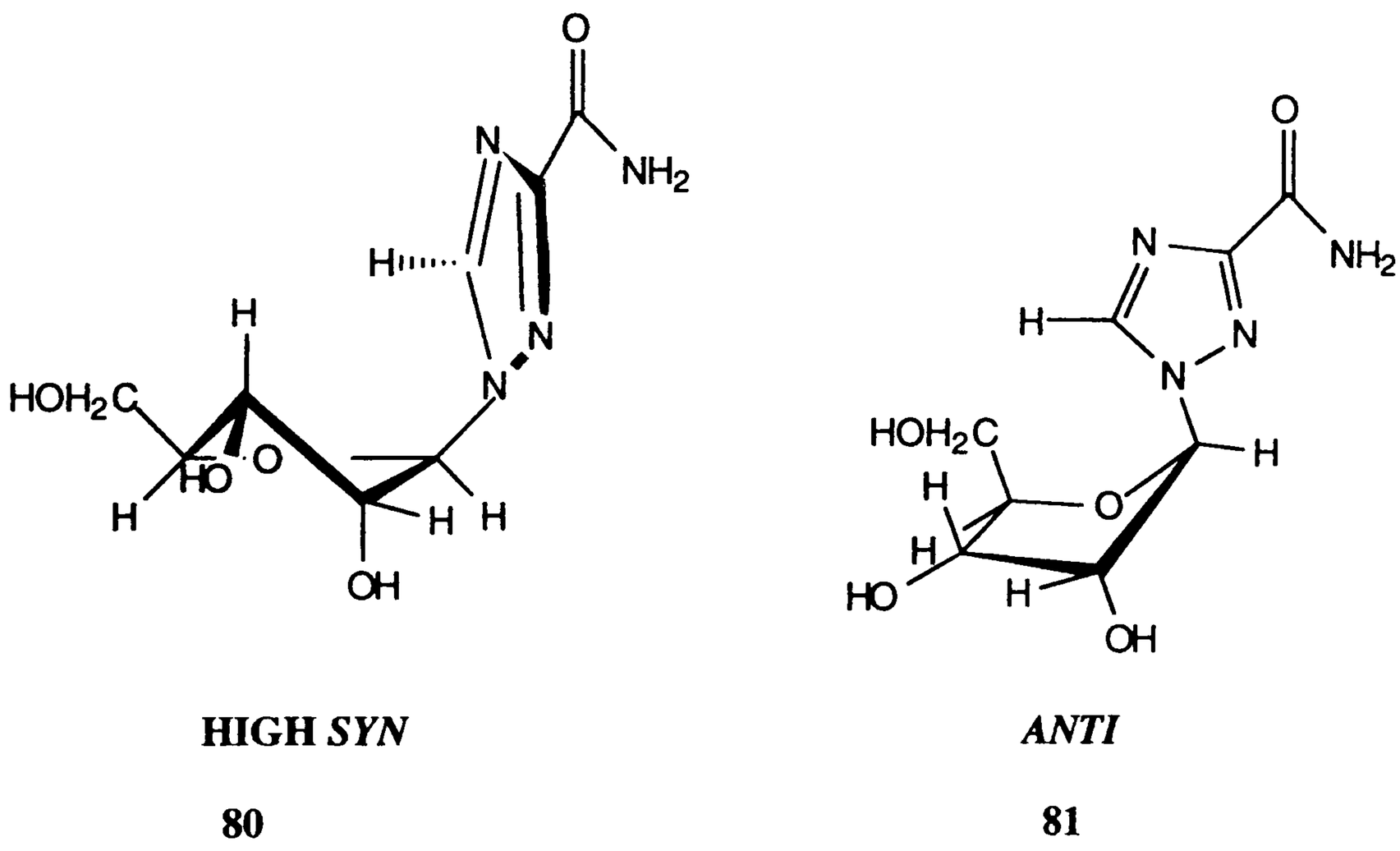
	Irradiation at H-1'			Irradiation at H-5
	% Enhancement at			
Compound	H-5	H-2'b (down)	H-4'	H-1'
65	4.35	—	1.16	5.66
66	3.00	6.20	—	—
67	2.40	4.50	1.00	2.90
68*	4.59	7.89	1.15	2.31
69*	6.25	7.42	1.56	6.02
70	2.01	3.27	1.12	4.40
71	—	6.43	1.17	—
72	—	6.50	1.10	—

* D_2O as solvent

Table 3.3 Nuclear Overhauser enhancements (%) caused by irradiating signals in the ^1H NMR spectrum of the 2'-deoxyribonucleoside analogues of ribavirin

As the triazole ring is on the topside of the glycosyl ring, enhancements of the signals due to H-2'a and H-3'a would be expected on irradiation of H-5 on the triazole ring.[213] This lack of enhancement implies that the

proton on the triazole ring is not positioned over the sugar moiety, in the *anti* conformation **80** (Fig. 3.4). In fact, H-5 is close to the H-2'a sitting over the top side of the glycosyl ring in the high *syn* conformation **81** (Fig. 3.4). As previously mentioned in Chapter One, theoretical studies have shown that ribavirin is in a high *syn* **81** conformation in solution. It appears that the conformation of these novel nucleosides adopted in solution remains the same as ribavirin.

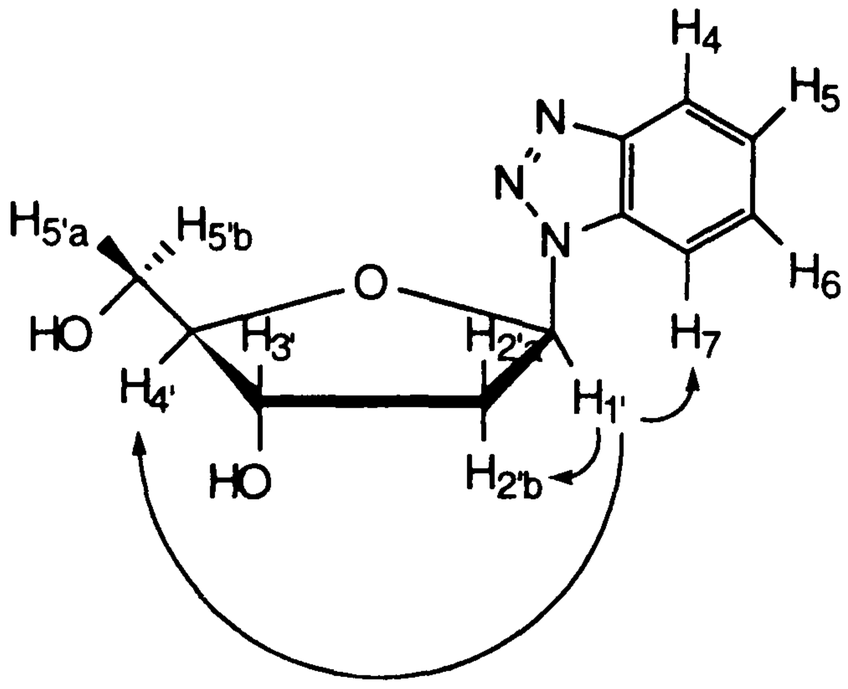


HIGH *SYN* ***ANTI***

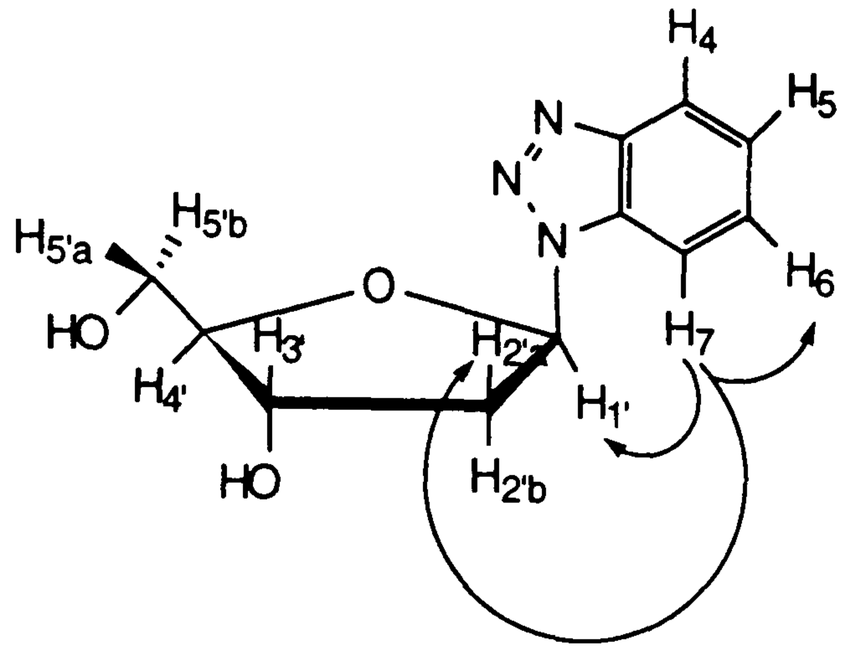
80 **81**

Fig. 3.4 Two conformations of ribavirin

The structure of the benzotriazole-2'-deoxynucleoside was confirmed by comparison of ^{1}H and ^{13}C NMR and nOe data previously published (Fig. 3.5a) (Table 3.4).[202]



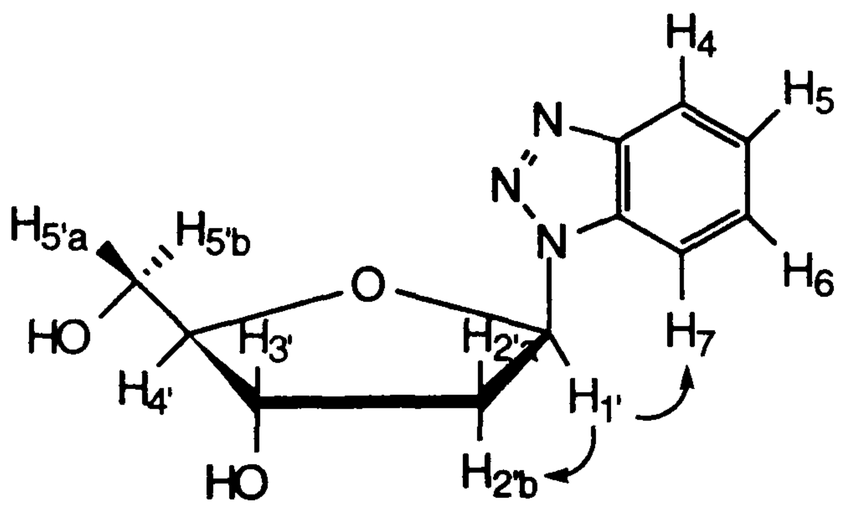
Irradiation at H-1'



Irradiation at H-7

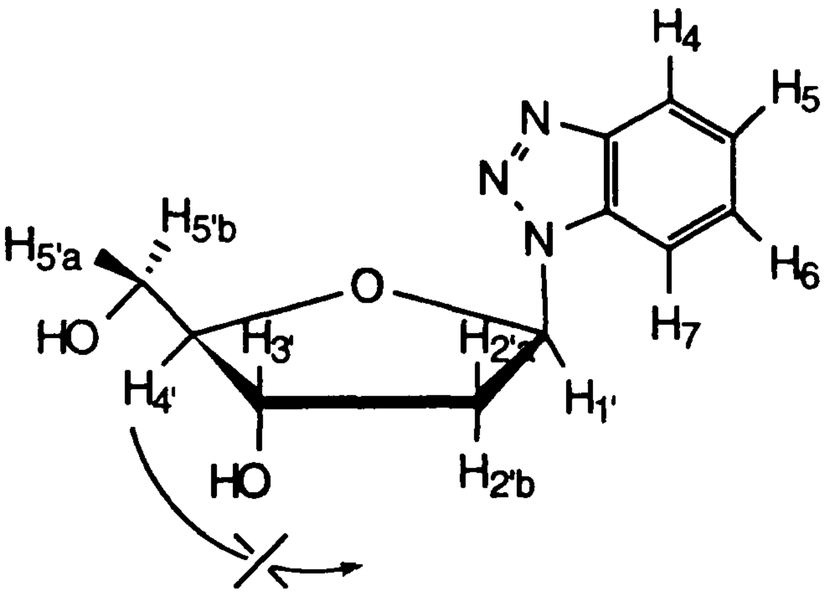
Fig. 3.5a Sites of nuclear Overhauser enhancements in the ^1H NMR spectrum of benzotriazole-2'-deoxynucleoside in DMSO-d^6

When the nOe is measured in D_2O, no enhancement is seen of the signal due to H-4' **83** (Fig. 3.5b). A signal is seen at H-2'b, which is on the underside of the ring (Table 3.4).



Irradiation at H-1'

82



Irradiation at H-4'

83

Fig. 3.5b Sites of nuclear Overhauser enhancements in the ^1H NMR spectrum of benzotriazole-2'-deoxynucleoside D_2O

This appears to break the rules stated previously, that irradiation at H-1'

will always produce enhancement at the signal due to H-4'. Exceptions to this rule are nucleosides with *O*-4'-exo conformation of the glyconic moiety.[212] Maybe the nucleoside adopts a different conformation in water to that in DMSO.

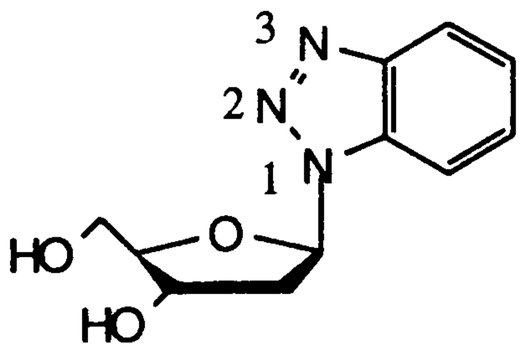
Irradiation at H-1' of **73**

	% Enhancement at		
Solvent	H-7	H-2'b	H-4'
DMSO-d^{6}*	6.0	7.0	1.6
DMSO-d^{6}	5.3	7.5	1.4
D_2O	4.7	6.5	—

* Figures from Kazimierczuk *et al*[202]

Table 3.4 Nuclear Overhauser enhancements (%) caused by irradiating signals in the ^{1}H NMR spectrum of the 2'-deoxyribonucleoside of benzotriazole

The position of glycosylation was established by nOe experiments. Due to the symmetry of benzotriazole there are only two possible products of the *N*-deoxyribosyltransferase reaction, substitution of the 2'-deoxyribose at N-1 **73** or N-2 **84** of the benzotriazole base (Fig. 3.6).



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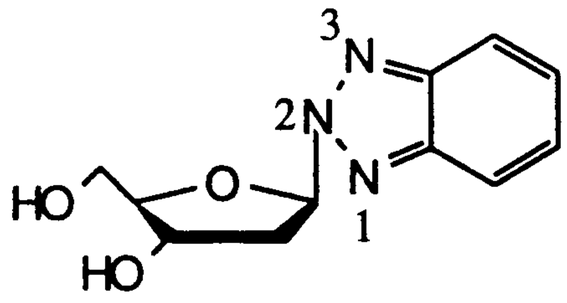


84

Fig. 3.6 Two possible sites of glycosylation

Glycosylation at N-2 **84** is known to give no enhancement at H-7 on irradiation of H-1',[202] so the sugar moiety must be attached at N-1 **73** (Fig. 3.6).

Inhibition Studies

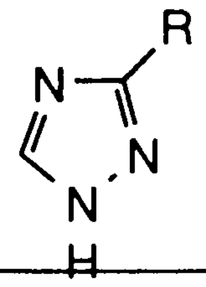
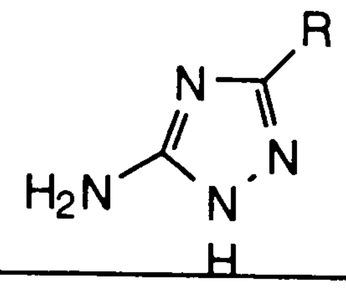
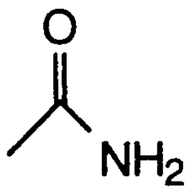
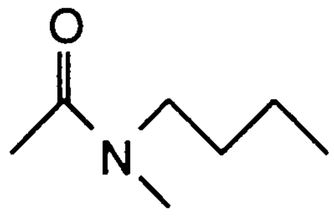
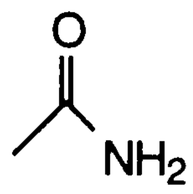
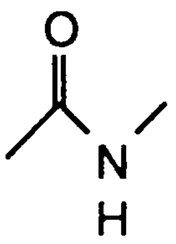
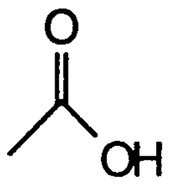
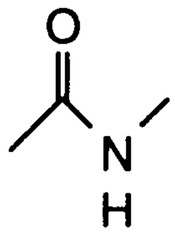
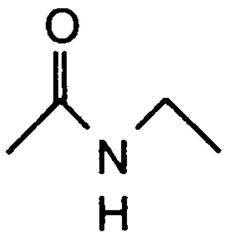
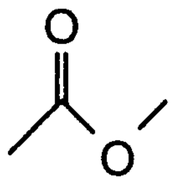
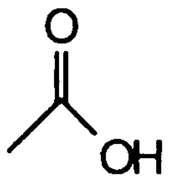
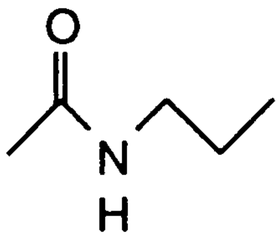
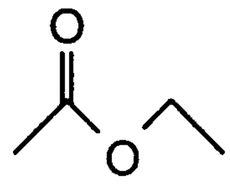
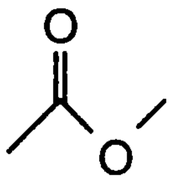
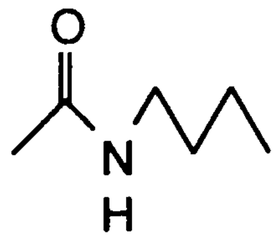
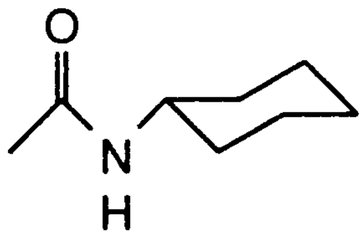
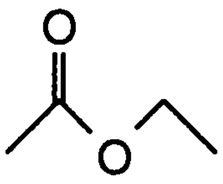
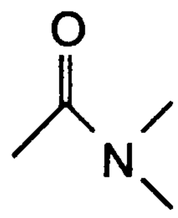
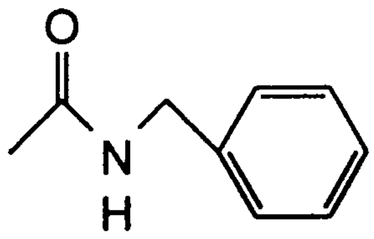
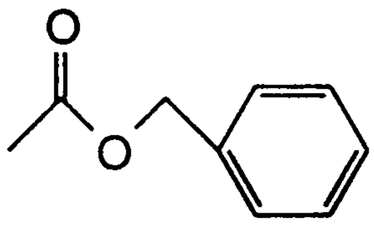
The possible inhibitory effects of two triazole bases ethyl-5-hydroxy-1,2,3-triazole-4-carboxylate **55** and *N*-ethyl-1,2,3-triazole-4-carboxamide **39** (Fig. 3.7) were investigated by incubating the compound with the crude *N*-deoxyribosyltransferase and then assaying the enzyme for activity after certain intervals of time. No loss in activity was observed so inhibition did not appear to be a factor for the lack of transfer.

55 **39**

Fig. 3.7 Triazole bases used in inhibition studies

Discussion

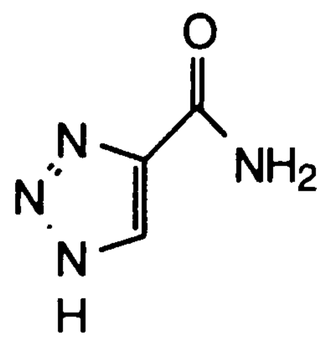
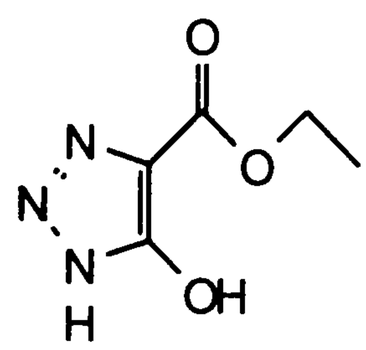
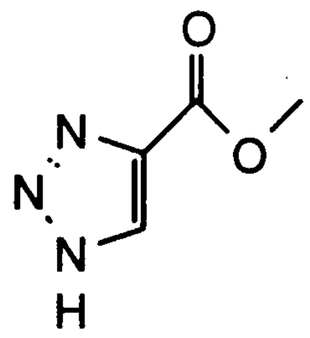
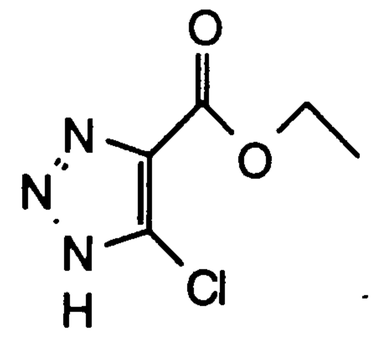
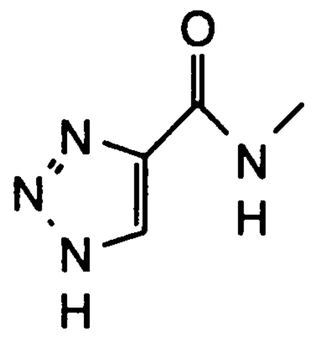
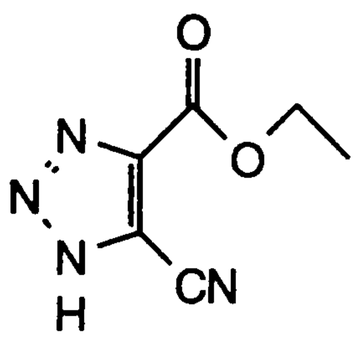
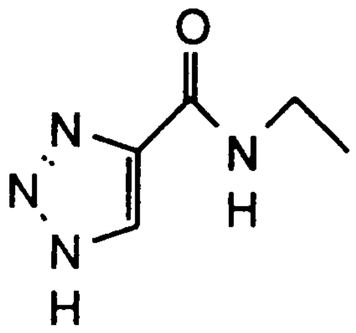
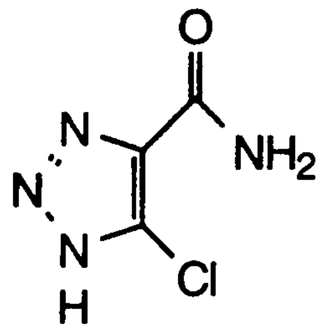
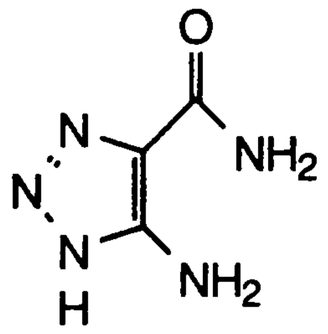
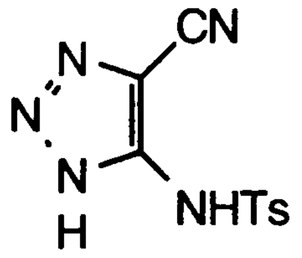
The following three tables (Tables 3.5, 3.6, 3.7) show the triazole bases synthesised and their success as acceptors in the *N*-deoxyribosyltransferase reaction.

									
R =						**R =**			
18		✓	**26**		✓	**13**		✓
19		✓	**15**		✗	**14**		✓
20		✓	**16**		✗	**10**		✗
21		✓	**17**		✗	**11**		✗
22		✓	**23**		✗	**12**		✗
25		✓	**24**		✗	**27**		✗

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.5 Results with 1,2,4-triazole bases used as acceptors in the *N*-deoxyribosyltransferase reaction

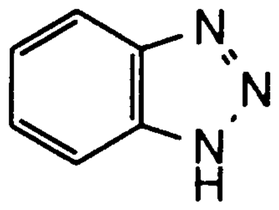
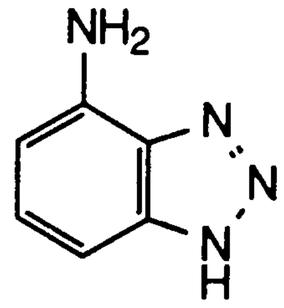
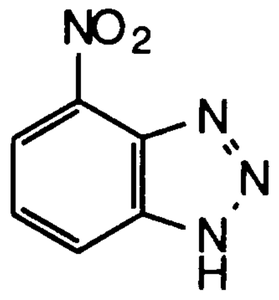
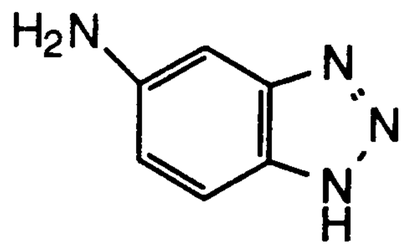
30		✓	55		✗
35		✗	59		✗
38		✗	56		✗
39		✗	58		✗
60		✓	48		✗

Ts = $CH_3C_6H_4SO_2$-

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.6 Results with 1,2,3-triazole bases used as acceptors in the *N*-deoxyribosyltransferase reaction

61		✓	64		✗
63		✗	87		✗

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.7 Results with benzotriazole bases used as acceptors in the *N*-deoxyribosyltransferase reaction

The discussion will be divided into three parts:

- an outline of the specificity studies carried out by other workers on *N*-deoxyribosyltransferases from *Lactobacillus helveticus* and *L. leichmannii*,
- a discussion on the previous work done by Hutchinson *et al* on the *N*-deoxyribosyltransferases from *L. leichmannii*,
- the results of the triazole bases in the *N*-deoxyribosyltransferase reaction.

Specificity Studies on *N*-Deoxyribosyltransferases from *Lactobacillus helveticus* and *L. leichmannii*

As previously discussed in the introduction in Chapter One, the *N*-deoxyribosyltransferases from *Lactobacillus helveticus* and *L. leichmannii* have been purified. The substrate specificities for the bases and the sugars have been investigated.

Two distinct enzymes were characterised, *N*-deoxyribosyltransferase I and *N*-deoxyribosyltransferase II (Fig. 3.8).

N-Deoxyribosyltransferase I

dRib-Pur(1) + Pur(2) ⇌ dRib-Pur(2) + Pur(1)

N-Deoxyribosyltransferase II

dRib-Pur(1) + Pur(2) ⇌ dRib-Pur(2) + Pur(1)

dRib-Pur + Pyr ⇌ dRib-Pyr + Pur

dRib-Pyr(1) + Pyr(2) ⇌ dRib-Pyr(2) + Pyr(1)

dRib = 2-deoxy-D-ribose

Pur = Purine

Pyr = Pyrimidine

Fig. 3.8 Activities shown by the two transferase enzymes

Holguin *et al*[97] concluded that (Fig. 3.9):

- the positions 1, 2 and 6 are of minor importance,
- only minor modifications can be tolerated on the imidazole ring,
- a tautomeric proton must be present on the imidazole moiety,
- the position of the tautomeric proton governs the site of substitution,
- for steric reasons, no substituent is allowed on position 8.

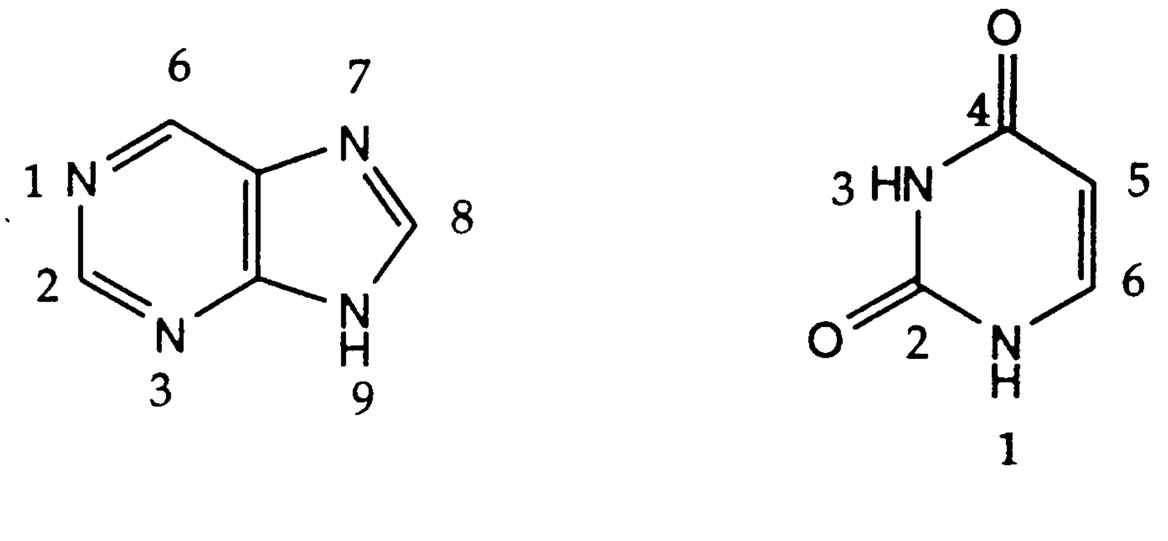
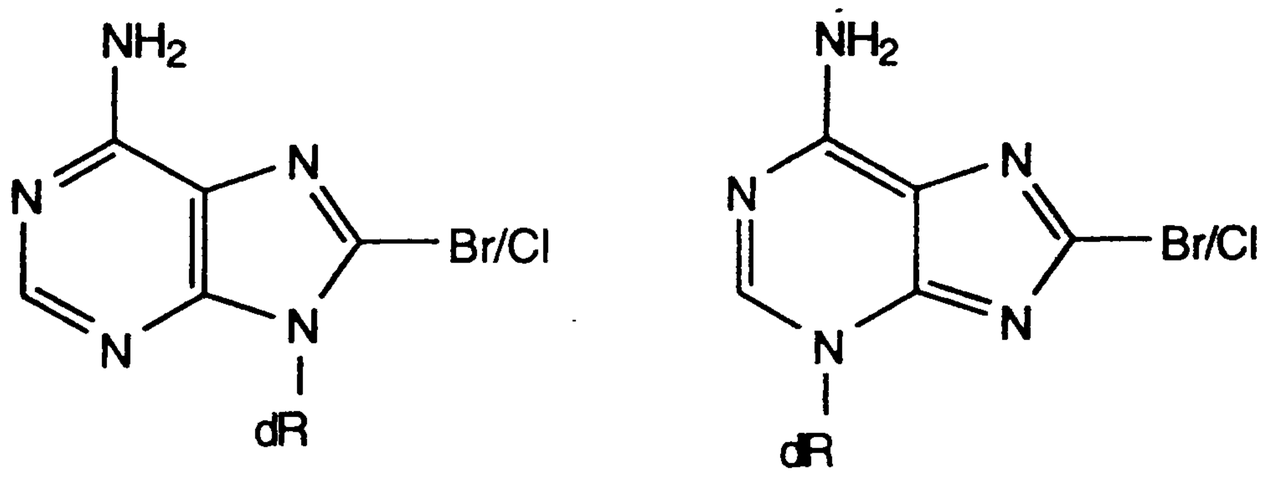


Fig. 3.9 Purine and pyrimidine bases

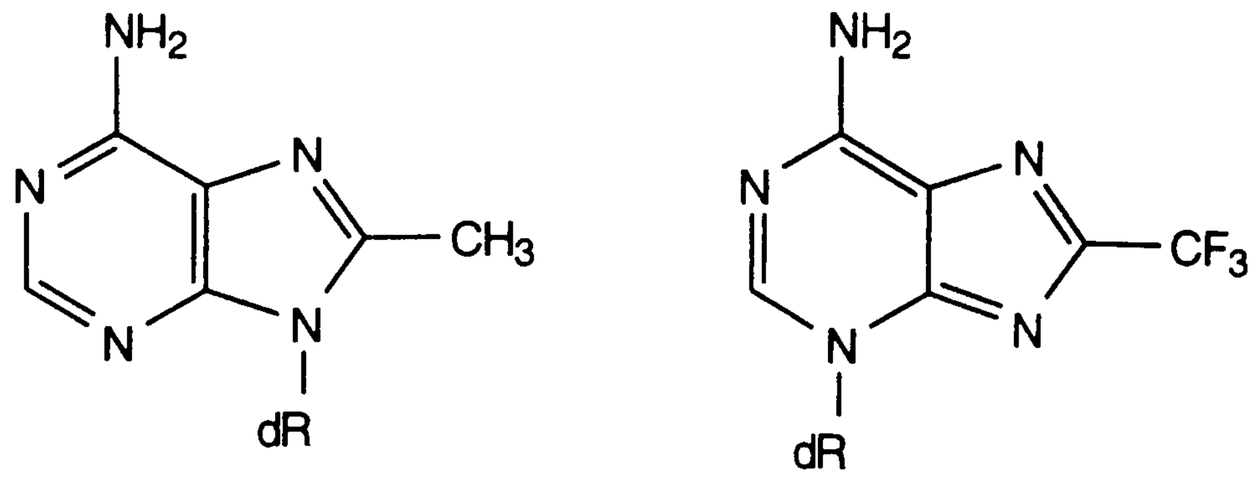
The same rules applied to the *N*-deoxyribosyltransferases isolated from *L. leichmannii*.[91, 107, 110] However, some purines substituted at position 8 did act as acceptors for the enzyme (Fig. 3.10 and 3.11).[110] The reaction rates were much slower and, in some cases, two products were formed. The formation of the 3-linked product was thought to be associated with the electronegativity of the substituent in the purine ring rather than the size of the group. 8-Methyl adenine gave solely the 9-glycosylated product, whereas replacement with the bromo or chloro group gave a mixture of the 3- and 9-glycosylated nucleosides (Fig. 3.10).



dR = 2'-deoxyribose

Fig. 3.10 Two products formed in the *N*-deoxyribosyltransferase reaction

The trifluoromethyl group, the most electronegative substituent, gave only the 3-glycosylated product (Fig. 3.11).

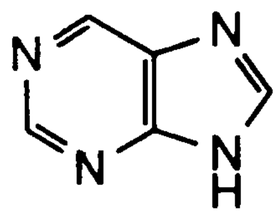
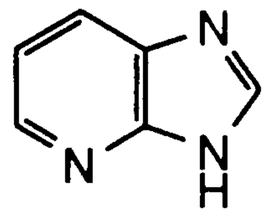
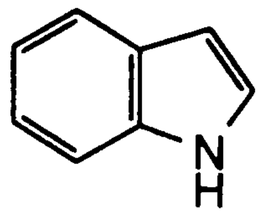
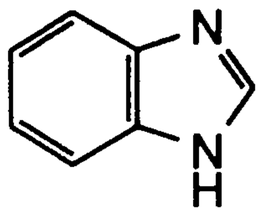
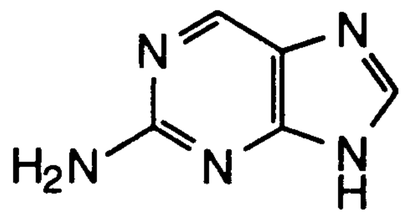
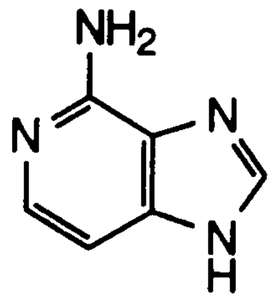
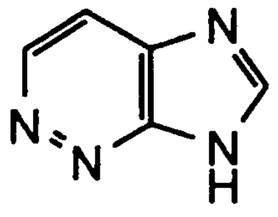
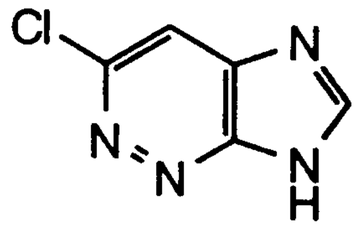
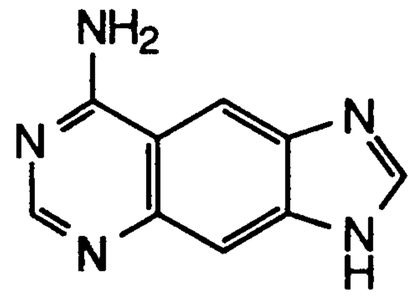
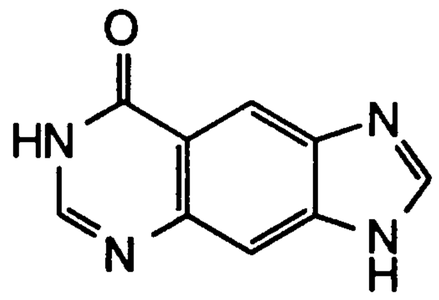
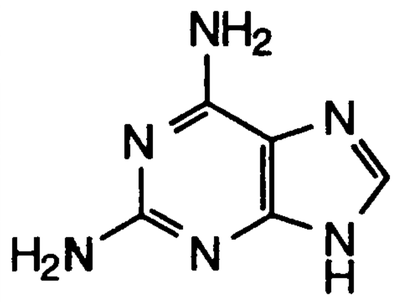
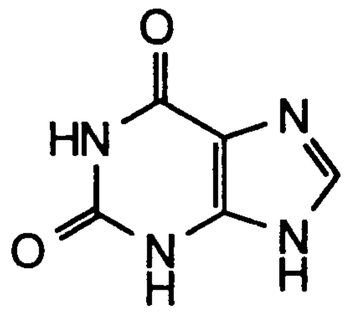


dR = 2'-deoxyribose

Fig. 3.11 Different *N*-glycosylated products in the *N*-deoxyribosyltransferase reaction

Previous Work Carried Out by Hutchinson *et al* on *N*-Deoxyribosyltransferases from *Lactobacillus leichmannii*

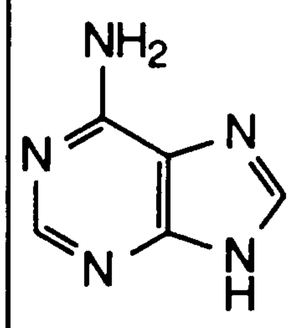
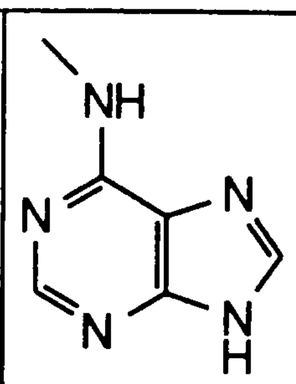
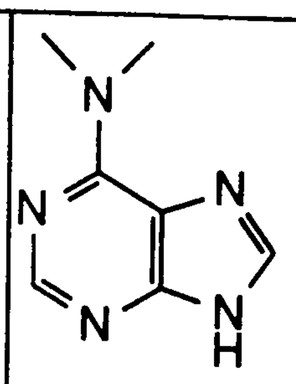
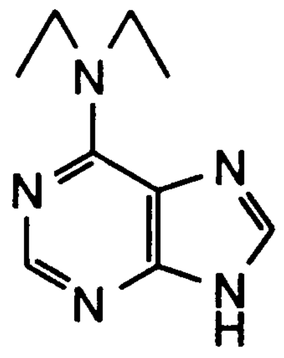
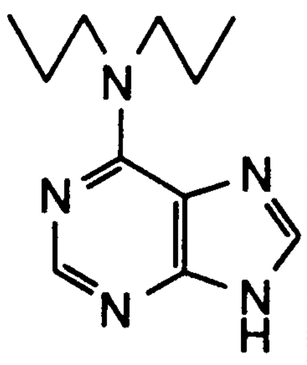
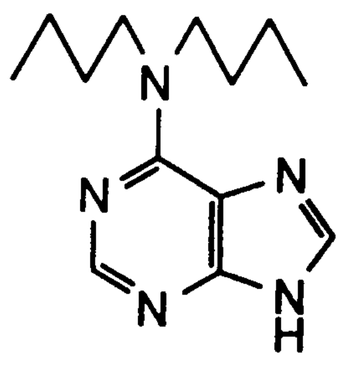
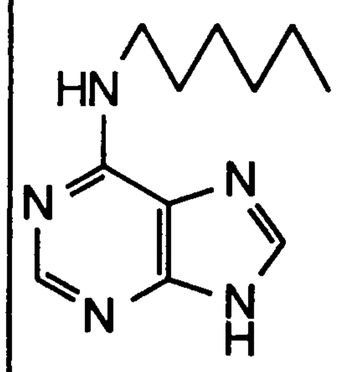
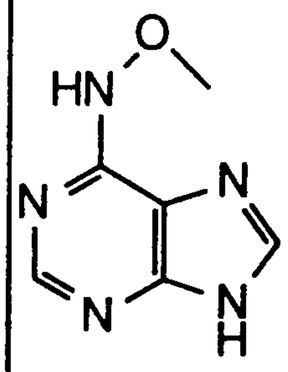
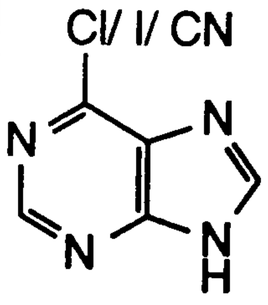
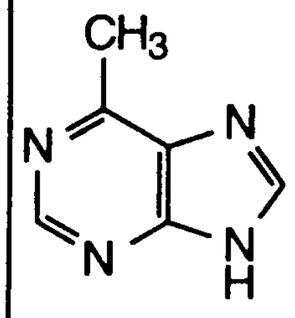
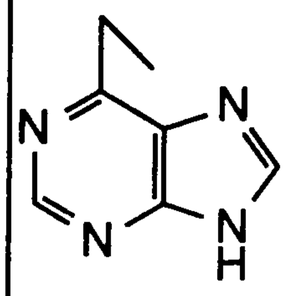
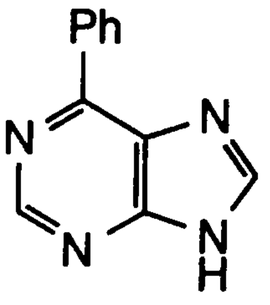
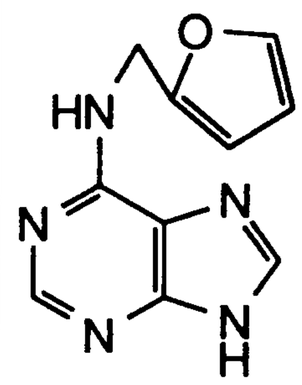
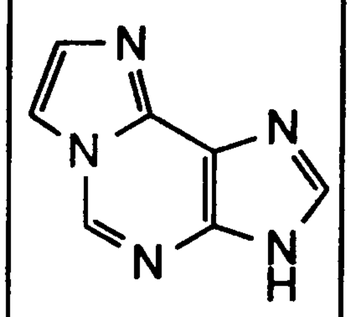
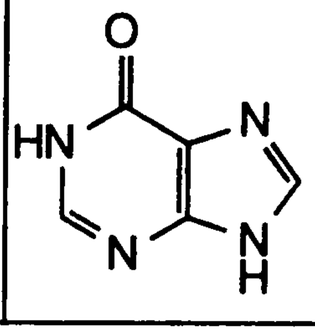
Two *N*-deoxyribosyltransferase activities have been isolated from *Lactobacillus leichmannii* by Hutchinson *et al*, as mentioned in Chapter One.[98] The substrate specificities of the *N*-deoxyribosyltransferases will be discussed in the following groups: modified purine bases, imidazole bases, modified pyrimidine bases and finally, the triazole bases. The Michaelis constants determined were 4 μM and 10 μM in the transfer reaction for *N*-deoxyribosyltransferases I and II, respectively.[98] The work on the modified purine bases, imidazole bases and modified pyrimidine bases has been previously published or is about to be published by a number of workers, all part of this group. The following seven tables (Tables 3.8-3.14) summarise their findings.[65, 98, 108, 109, 111, 206, 214-217]

88		✓	92		✓	97		✗
62		✓	93		✓	98		✗
89		✓	94		✓			
90		✓	95		✓			
91		✓	96		✓			

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

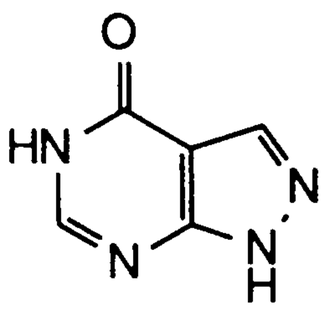
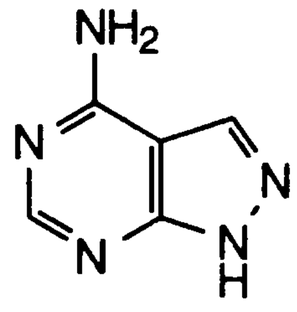
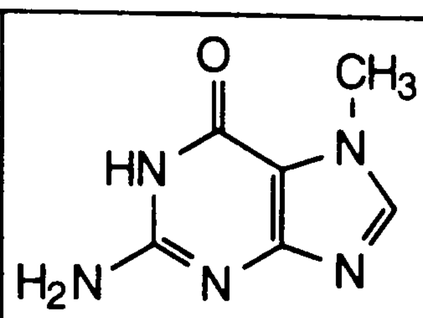
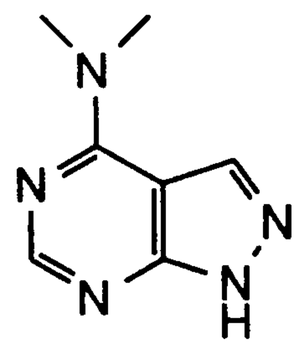
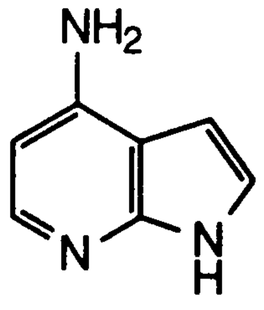
Table 3.8 Modification on position 1, 2 and 3 of the purine ring[65, 98, 108, 109, 206, 214, 216]

99		✓	104		✓	109		✓
100		✓	105		✓	110		✓
101		✓	106		✓	111 112 113		✓
102		✓	107		✓	114		✓
103		✓	108		✓	115		✓

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

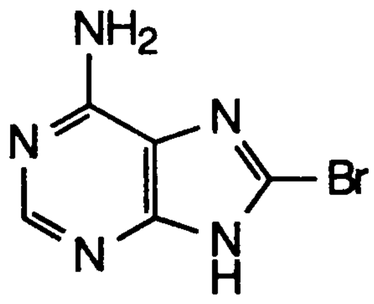
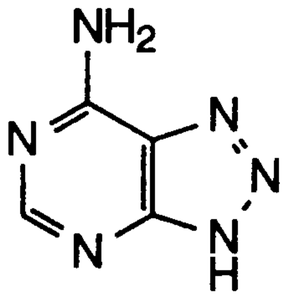
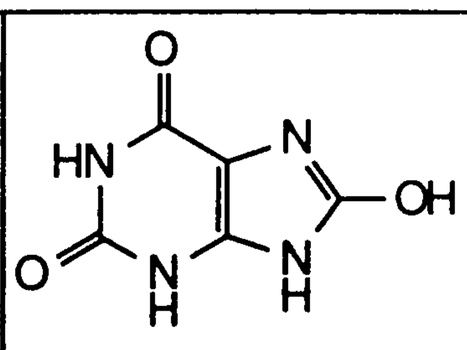
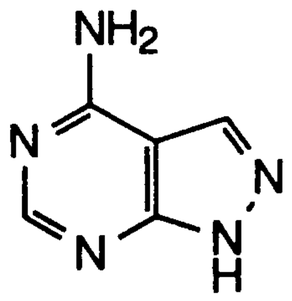
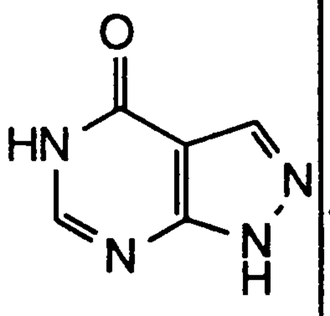
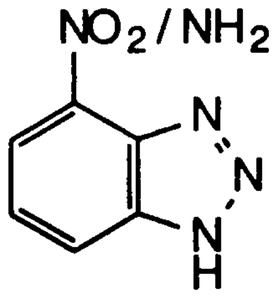
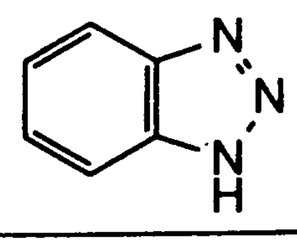
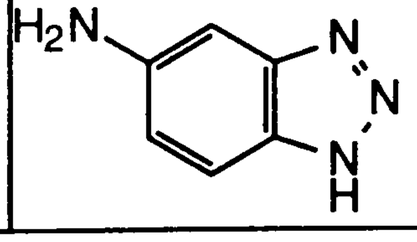
Table 3.9 Modification on position 6 of the purine ring[65, 98, 109, 114, 216]

116		✓	**118**		✓	**119**		✗
117		✓				**120**		✗

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

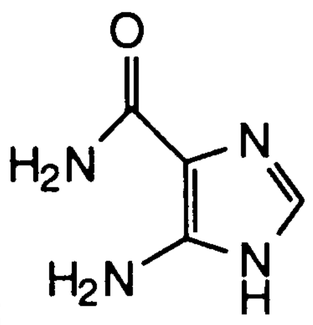
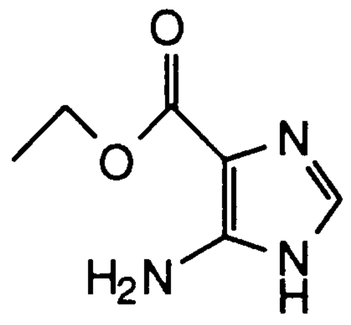
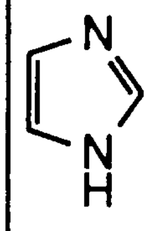
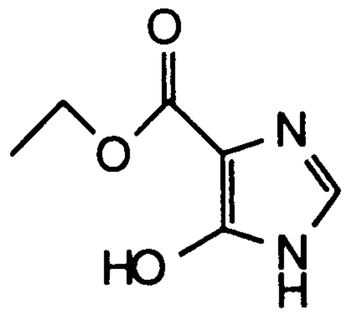
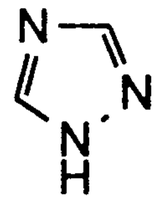
Table 3.10 Modification on position 7 of the purine ring[98, 109]

121		✓	**123**		✓	**125**		✗
122		✓	**124**		✓	**63** **64**		✗
61		✓				**87**		✗

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

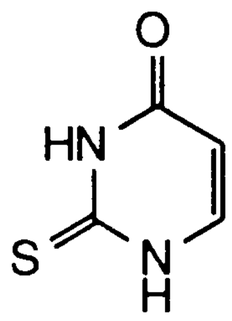
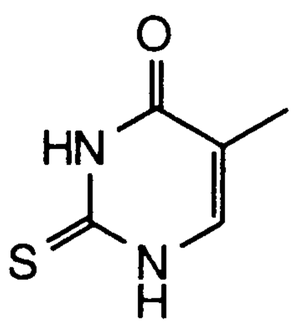
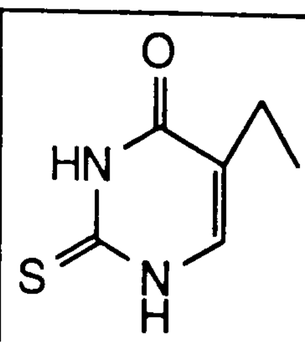
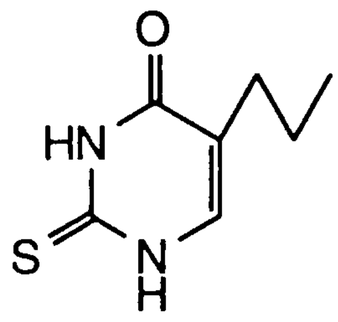
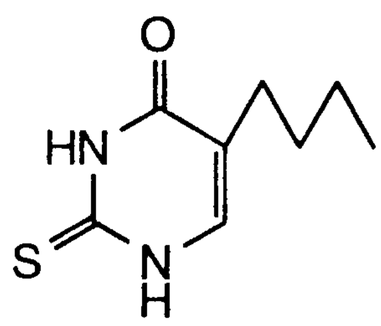
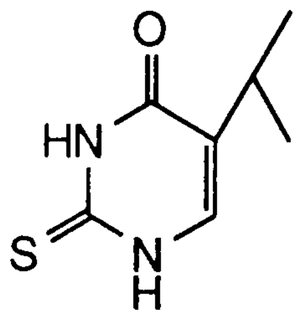
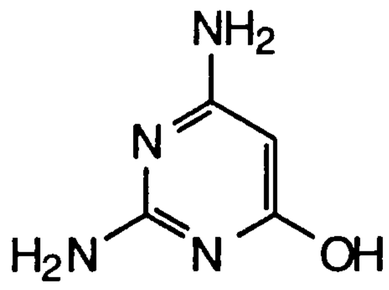
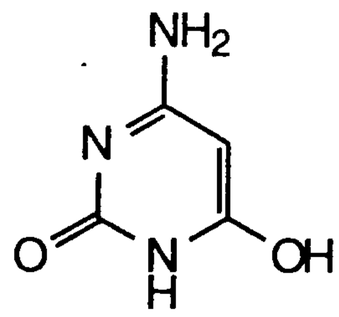
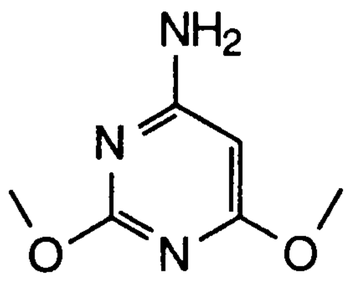
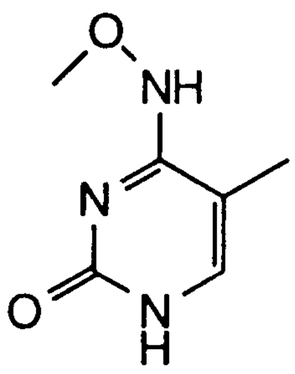
Table 3.11 Modification on position 8 of the purine ring[98, 109, 217]

126		✓	**128**		✓	**130**		✗
127		✓	**129**		✓			

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

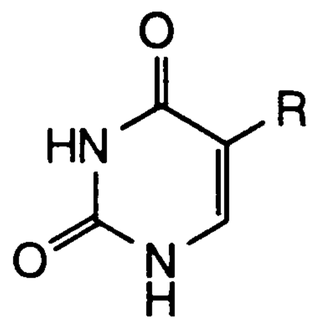
Table 3.12 Modification of the purine ring[98, 111]

131		✗	135		✗	139		✗			
132		✗	136		✗	140		✗			
133		✗	137		✗						
134		✗	138		✗						

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.13 Modification on positions 2, 4, 5 and 6 of the pyrimidine ring[98, 206]



R =

141	H	✓	**146**	CH_3	✓	**151**		✗
142		✓	**147**	F	✓	**152**		✗
143	Cl	✓	**148**	Br	✓	**153**		✗
144	I	✓	**149**	CF_3	✓	**154**	COOH	✗
145	NH_2	✓	**150**	NO_2	✓	**155**		✗

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

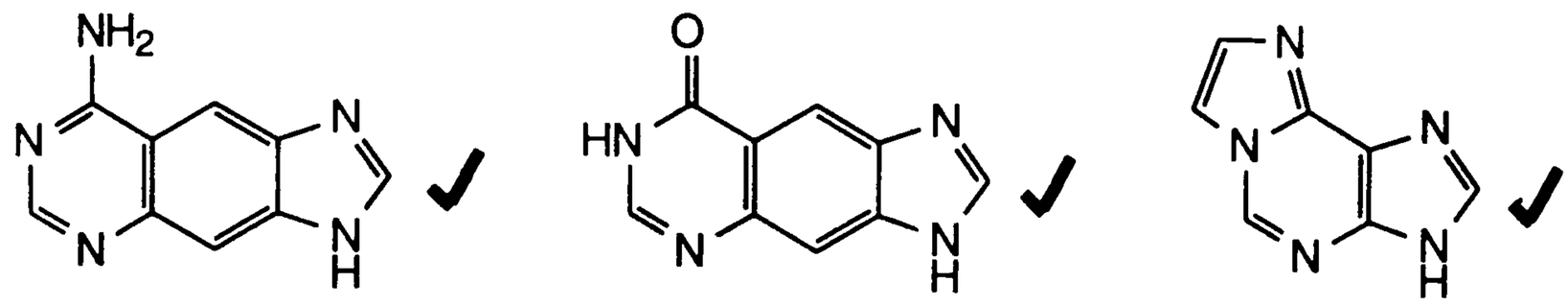
Table 3.14 Modification on position 5 of the pyrimidine ring[98]

In general, the rules previously established for the *N*-deoxyribosyltransferases from *L. helveticus*[97] were found to apply to the *N*-deoxyribosyltransferases from *L. leichmannii*.

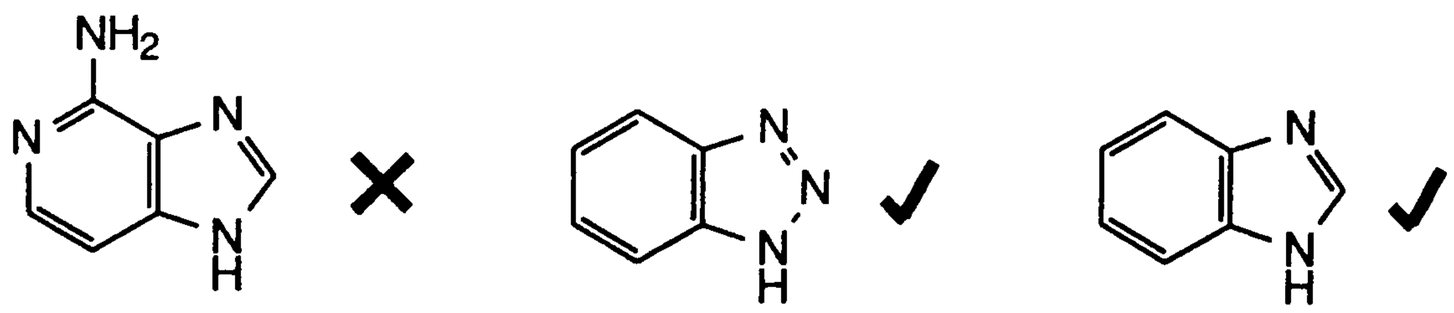
Modification of the Purine Ring at Positions 1, 2 and 3

The positions 1 and 2 of the purine ring can tolerate substitution of nitrogen for CH and *vice versa* (Table 3.8). A third ring can be accommodated, as in the tricyclic bases **90**, **95** and **108** (Fig. 3.12). However, changes at position 3 can result in bases that do not act as acceptors, such as 3-deazaadenine **98**, though other bases, benzotriazole **61** and benzimidazole **62**, lacking the nitrogen atom at position 3, are

acceptors (Fig. 3.12). Substitution at the 3 position has been reported to give either slow or non active substrates.[97]



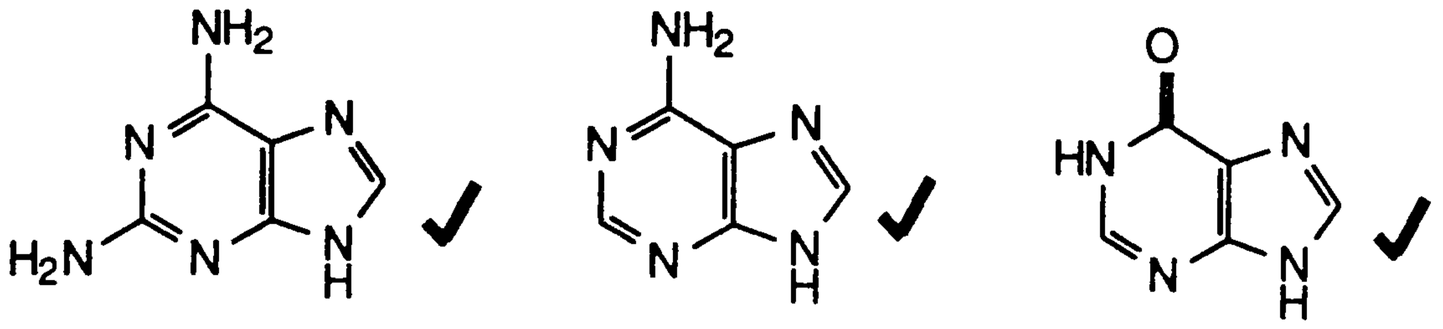
90 **95** **108**



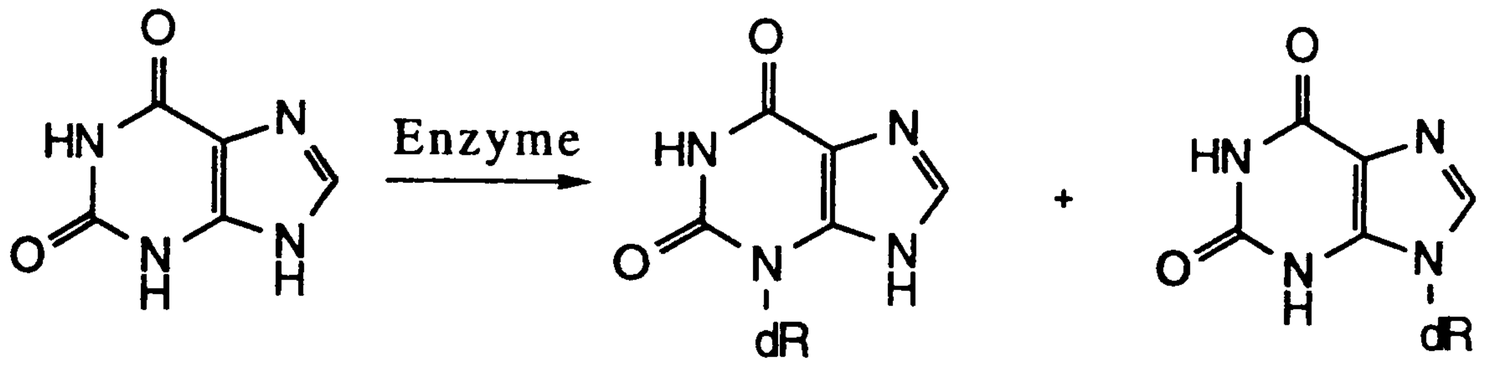
98 **61** **62**

Fig. 3.12 Bases modified at positions 1, 2 and 3 of the purine ring

The 2,6-disubstituted purines, diaminopurine **91** and xanthine **96** (Table 3.8) (Fig. 3.13) have slower velocities than the corresponding monosubstituted bases, adenine **99** and hypoxanthine **115** (Table 3.9). Xanthine **96** gave two products, thought to be the 3-β-D-deoxyribonucleoside and the 9-β-D-deoxyribonucleoside (Fig. 3.13). The base seeks an alternative access to normal binding, docking into the protein catalytic site *via* the pyrimidine rather than the purine ring.[98] Though the presence of a nitrogen at position 1 or 3 is not vital for the ability to act as a substrate, weak interactions could come into play between the nitrogens of the purine ring and the active site helping the base to bind to the active site. It would be interesting to see if tricyclic compounds lacking nitrogens in the ring are active as substrates.



91 **99** **115**

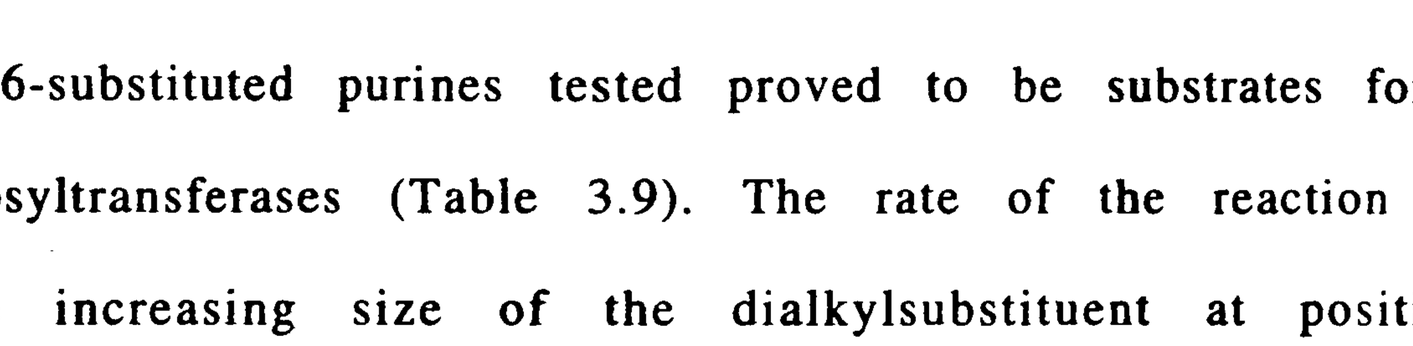


96 dR = 2-deoxyribose

Fig. 3.13 Comparison of 6-mono- and 2,6-disubstituted purine bases

Modification of the Purine Ring at Position 6

All the 6-substituted purines tested proved to be substrates for the *N*-deoxyribosyltransferases (Table 3.9). The rate of the reaction decreases with the increasing size of the dialkylsubstituent at position 6.[98] Replacement of the amino group, such as **104**, with the hydrophobic ethyl **107** or phenyl **114** group at position 6 does not significantly affect the reaction rate (Fig. 3.14).[215] These results show that the 1, 2 and 6 part of the purine ring resides in a large pocket in the active site.

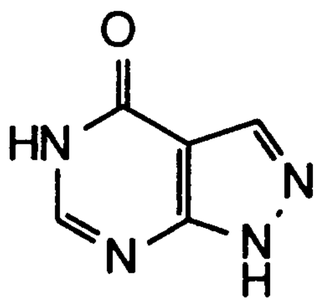
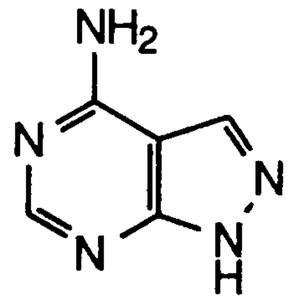
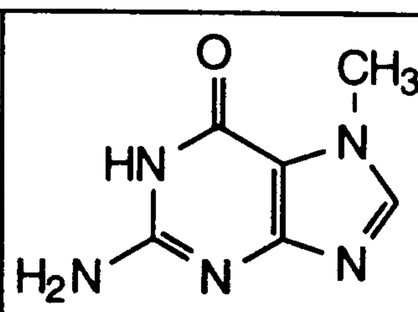
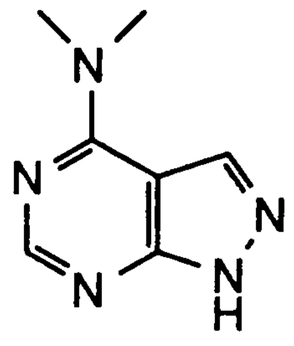
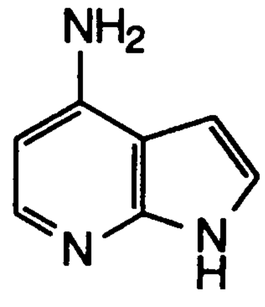


104 **107** **114**

Fig. 3.14 6-Substituted purine bases

Modification of the Purine Ring at Position 7

The results in Table 3.10 show that a tautomeric proton is required in the imidazole ring. The 7-substituted purines, 7-methylguanine **119** and 1,7-deazaadenine **120** are not acceptors where the tautomeric shift is not possible.[98] The nitrogen atom at N-7 is not essential for binding as the three 7-deaza-8-azapurines **116, 117** and **118** are acceptors.

116		✓	**118**		✓	**119**		✗
117		✓				**120**		✗

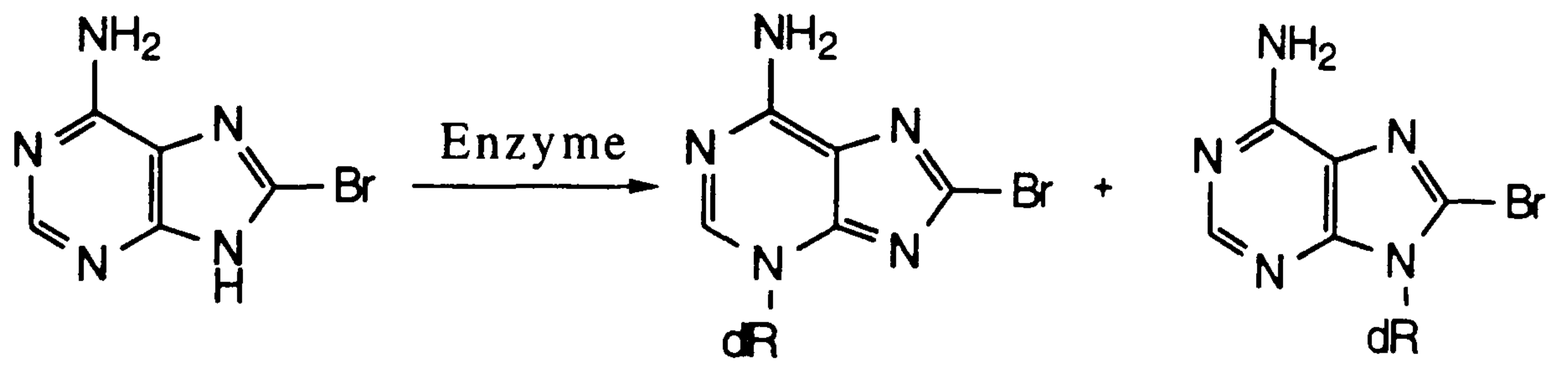
✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.10 Modification on position 7 of the purine ring

Modification of the Purine Ring at Position 8

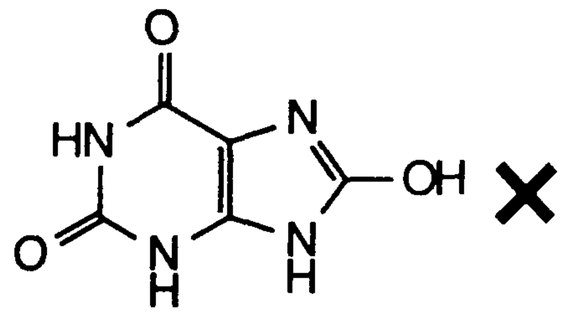
All but one of the 8-modified analogues tested were substrates (Table 3.11). 8-Bromoadenine **121** reacted very slowly and gave two products, probably the 3-β-D-deoxyribonucleoside and the 9-β-D-deoxyribonucleoside (Fig. 3.15),[98] which agrees with results published by Huang *et al.*[110]



121 dR = 2-deoxyribose

Fig. 3.15 The two possible products of the transferase reaction with 8-bromoadenine as acceptor base

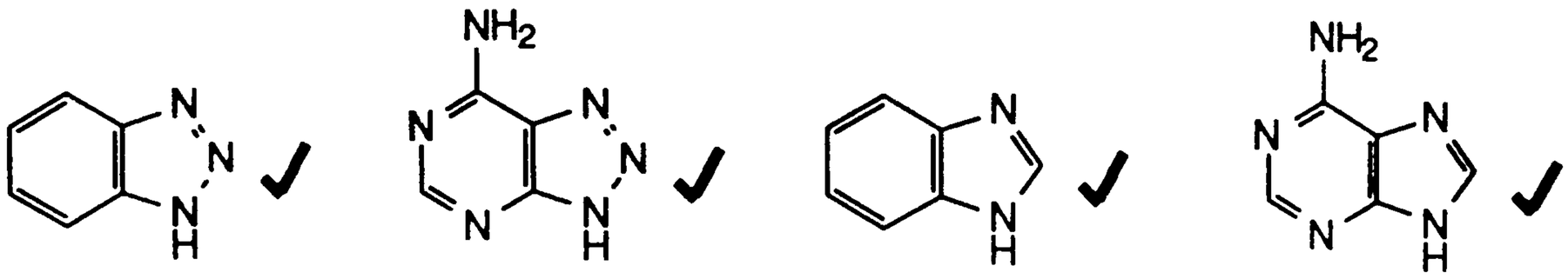
Uric acid **125** was not a substrate, which indicates that the 8-substituted analogues have difficulty in entering the active site due to steric hindrance (Fig. 3.16).



125

Fig. 3.16 Uric acid is not an acceptor in the *N*-deoxyribosyltransferase reaction

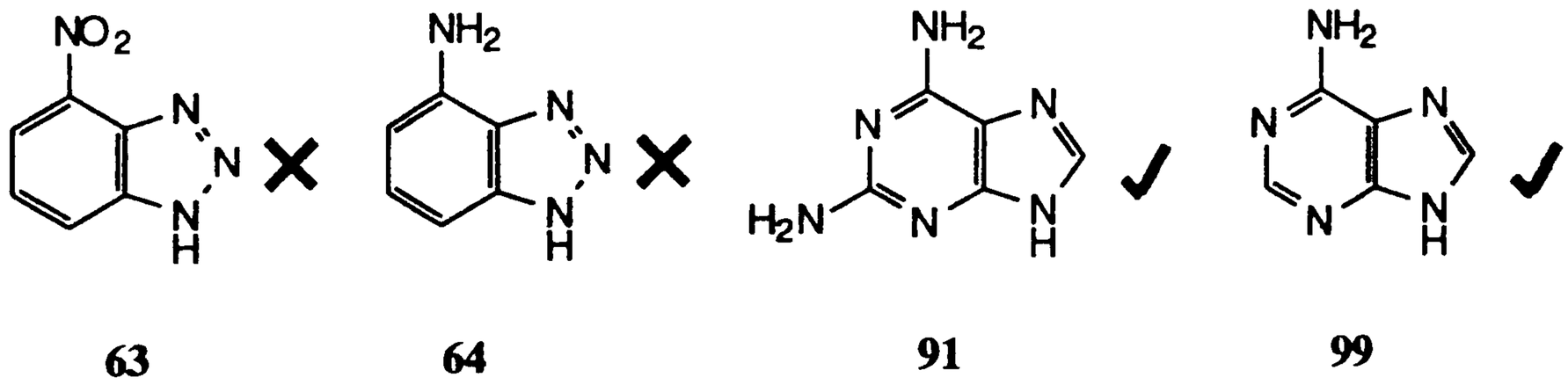
The 8-aza analogues, benzotriazole **61** and **123** were competent acceptors, but had reduced velocities compared to their 8-deaza analogues, benzimidazole **62** and adenine **99** (Fig. 3.17). Thus, though the presence of the extra nitrogen in the ring at position 8 does not prevent the base acting as an acceptor, it does hinder the reaction.



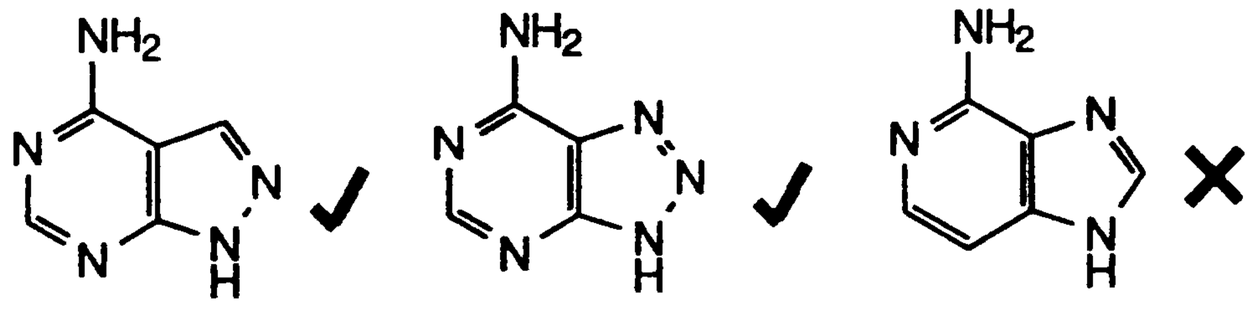
61 123 62 99

Fig. 3.17 Comparison of purine bases modified at position 8

It is unclear why the 4-nitrobenzotriazole **63** and 4-aminobenzotriazole **64** are not substrates in the transfer reaction. It has been shown that the active site is big enough to accommodate the amino and nitro groups (Table 3.9). Other 6-aminopurine analogues such as 2,6-diaminopurine **91**, adenine **99**, 7-deaza-8-azaadenine **118** and 8-azaadenine **123** are all acceptors, though 3-deazaadenine **98** is not (Fig. 3.18).



63 64 91 99



118 123 98

Fig. 3.18 Comparison of 6-amino-substituted purine bases modified at position 8

Benzotriazole **61** is an acceptor, even though the base lacks the nitrogens in the 6-membered ring and has an extra nitrogen at position 2

(the equivalent to position 8 in the purine ring). The presence of a substituent at position 4 of the benzotriazole base (the equivalent of the 6 position in the purine ring) **63**, **64**, coupled with the other changes within the ring, might put the molecule under some conformational strain. This prevents the 4-substituted benzotriazole from either entering the active site or being able to bind. The substituents at positions 4 and 5, for steric reasons, prevent the base from docking in the active site in the same way as the parent compound, benzotriazole **61**.

Modification of the Purine Ring

The incomplete purine structure can participate in the transfer reaction (Table 3.12). The three imidazole bases tested, 4-amino-imidazole-5-carboxamide **126**, ethyl-4-hydroxy-imidazole-5-carboxylate **127** and ethyl-4-amino-imidazole-5-carboxylate **128**, were all acceptors, though with a slower rate of transfer compared with the natural substrates.[98] The 1,2,4-triazole base **129** was found to be an acceptor, however the imidazole base **130** was not. Previous studies have found imidazole to be an inhibitor of the *N*-deoxyribosyltransferases.[97]

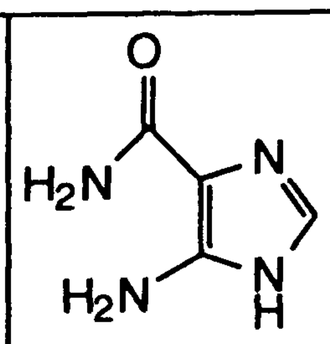
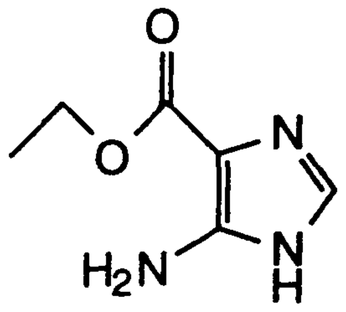
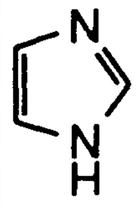
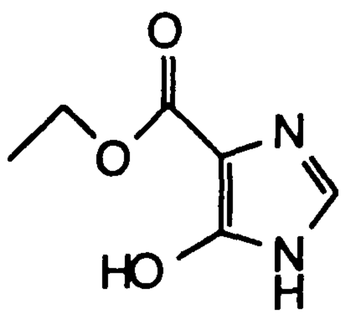
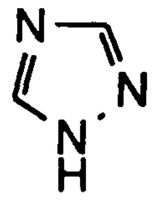
126		✓	**128**		✓	**130**		✗	
127		✓	**129**		✓				

Table 3.12 Modification of the purine ring

From these results the following rules can be established and a model of the active site proposed (Fig. 3.19):

Summary:

- positions 1, 2 and 6 are of little importance,
- large groups can be accommodated at position 6 of the purine ring,
- a tautomeric proton is necessary on the imidazole ring,
- only minor modifications can be tolerated at positions 3 and 8, for steric reasons,
- the imidazole moiety alone can act as an acceptor.

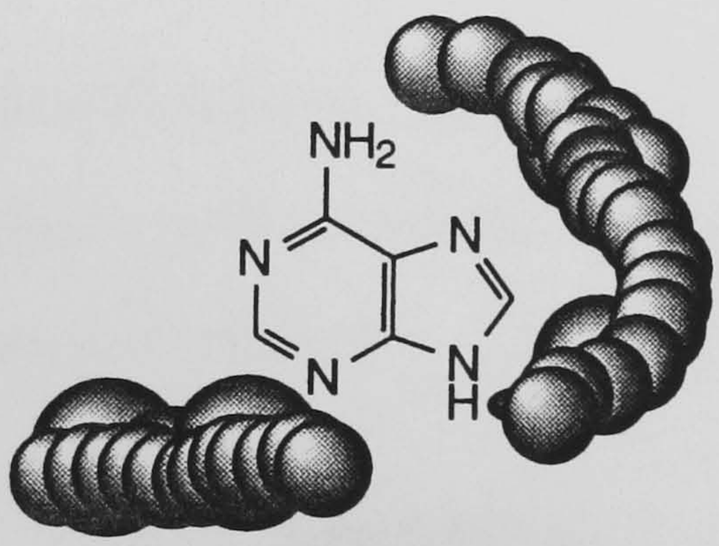


Fig. 3.19 Model of the enzyme active site for purine bases

Modification of Positions 2, 4, 5 and 6 of the Pyrimidine Ring

N-Deoxyribosyltransferase II has a much narrower specificity for the pyrimidine ring. Tautomerism is necessary between N-1 and N-3 of the pyrimidine ring (Fig. 3.20). Substitutions at position 2 and 6 result in non acceptors (Table 3.13). However, position 5 on the pyrimidines seems to be analogous to position 6 on the purines in regard to its versatility. Substituents such as halides and small alkyl groups were substrates. Any substituent on position 5 bigger than the ethyl group resulted in an inactive compound, for steric reasons.

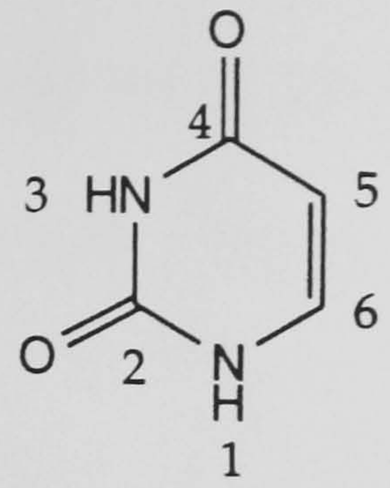


Fig. 3.20 Pyrimidine base

The following conclusions can be drawn from these results and a model of the active site proposed (Fig. 3.21):

Summary:

- positions 1, 2 and 6 are crucial for activity,
- tautomerism between positions 1 and 3 is necessary,
- small substituents at position 5 are tolerated.

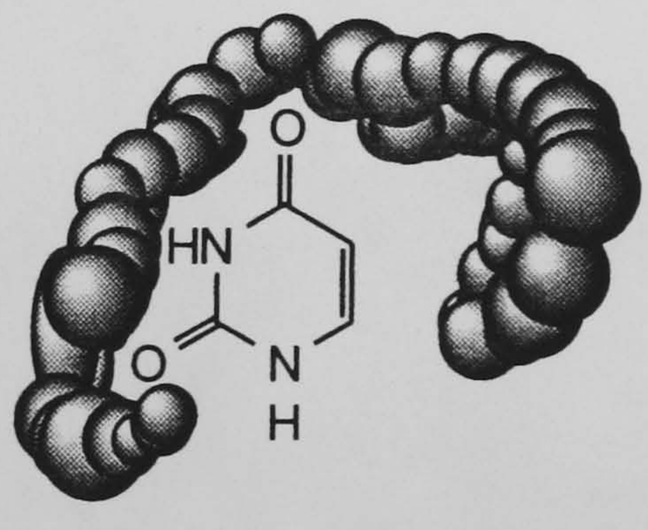


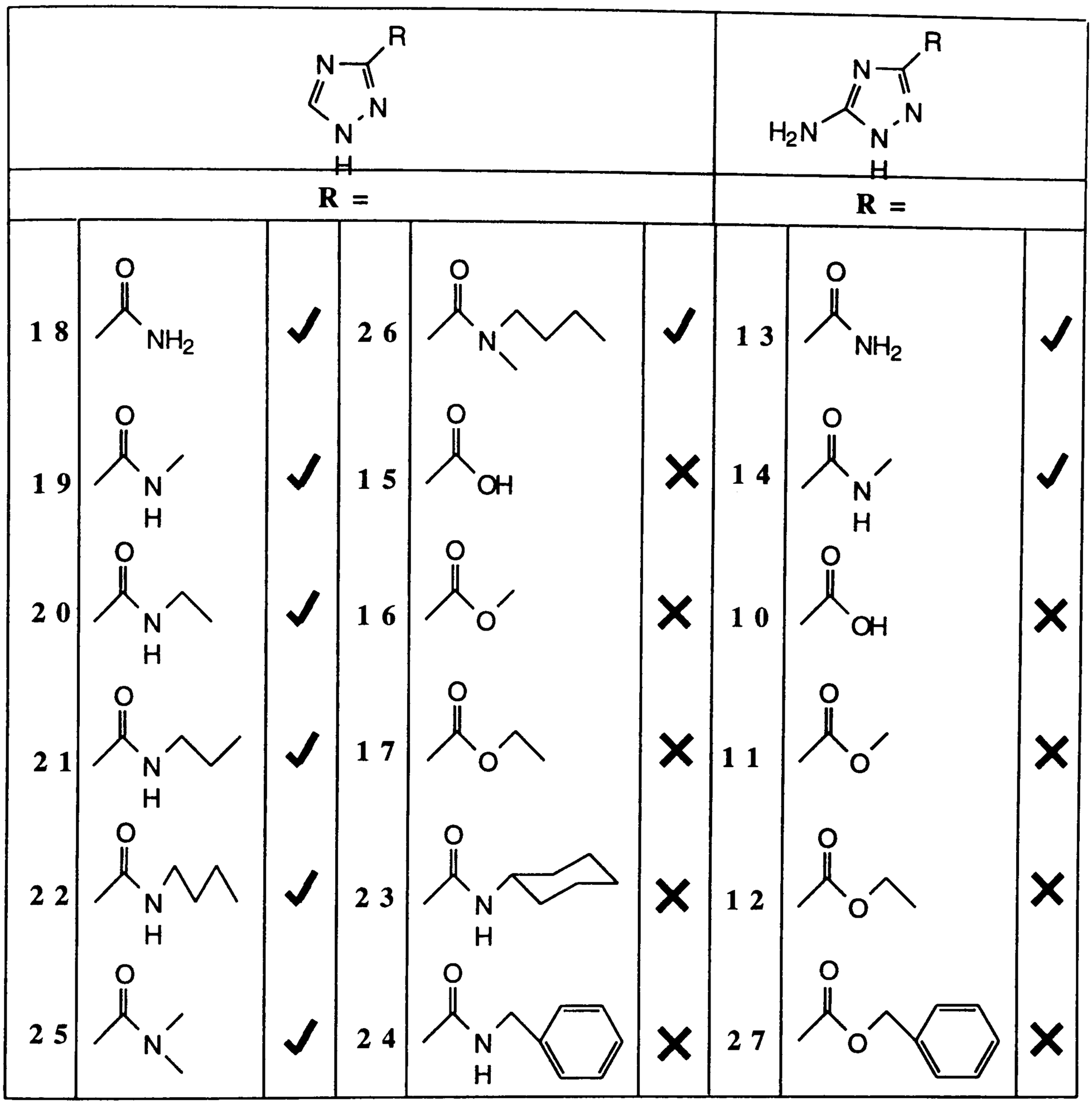
Fig. 3.21 Model of enzyme active site for pyrimidine bases

Results

Thirty two triazole bases were investigated as possible acceptors for the *N*-deoxyribosyltransferase from *Lactobacillus leichmannii*. Of these thirty two, twelve were found to act as acceptors and nine were isolated and fully characterised (Table 3.1).

1,2,4-Triazole Bases

Only the carboxamides of the 1,2,4-triazoles **13**, **14**, **18-22**, **25** and **26** synthesised were acceptors for the *N*-deoxyribosyltransferase (Table 3.5).



✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.5 Results with 1,2,4-triazole bases used as acceptors in the *N*-deoxyribosyltransferase reaction

The rate of the reaction decreased with the increasing size of alkyl group on the amide. The methyl **11** and **16**, ethyl **12** and **17** or benzyl **27** esters were not acceptors, nor were the carboxylic acids **10** and **15**. This led to

the conclusion that the hydrogen bonding properties of the amide were essential for binding. However, the *N*,*N*-dimethyl-1,2,4-triazole **25** was found to act as an acceptor and the nucleoside **70** was isolated. The rates of transfer of the *N*-butyl-1,2,4-triazole-3-carboxamide **22** and *N*-methyl,*N*-butyl-1,2,4-triazole-3-carboxamide **26** and were compared and the rates were found to be the same. This proved that the amide proton was not essential for binding. Replacing the amide with an ester or carboxylate group led to an inactive base. The nitrogen atom must be necessary for binding, anchoring the base in the active site. Loss of the amide group might prevent the triazole ring from docking correctly in the active site.

Not all the 1,2,4-triazole-3-carboxamides synthesised acted as acceptors. Alkyl substituents on the carboxamide of the 1,2,4-triazole base as big as n-butyl **22** were acceptors in the *N*-deoxyribosyltransferase reaction. Steric factors might explain the lack of transfer with *N*-benzyl-1,2,4-triazole-3-carboxamide **24** and *N*-cyclohexyl-1,2,4-triazole-3-carboxamide **23**. Previous studies with *N*-deoxyribosyltransferases from *Lactobacillus helveticus* have shown that bases with bulkier substituents at position 6 can act as acceptors (Fig. 3.22).

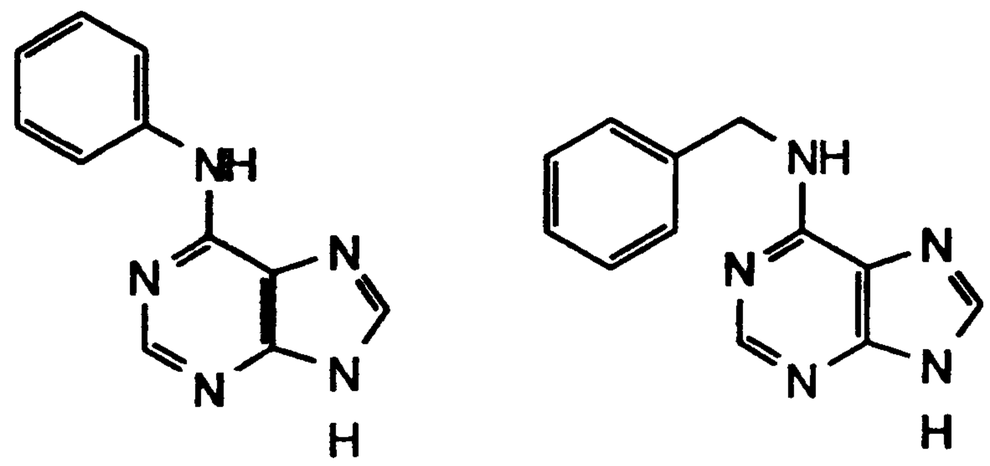
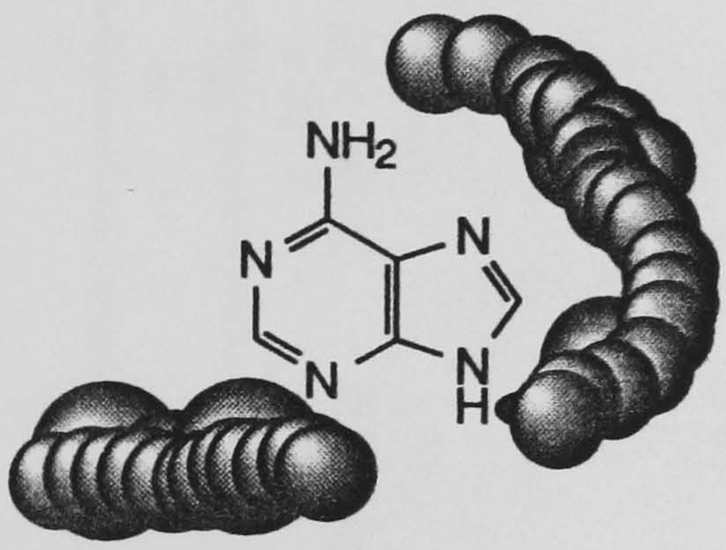
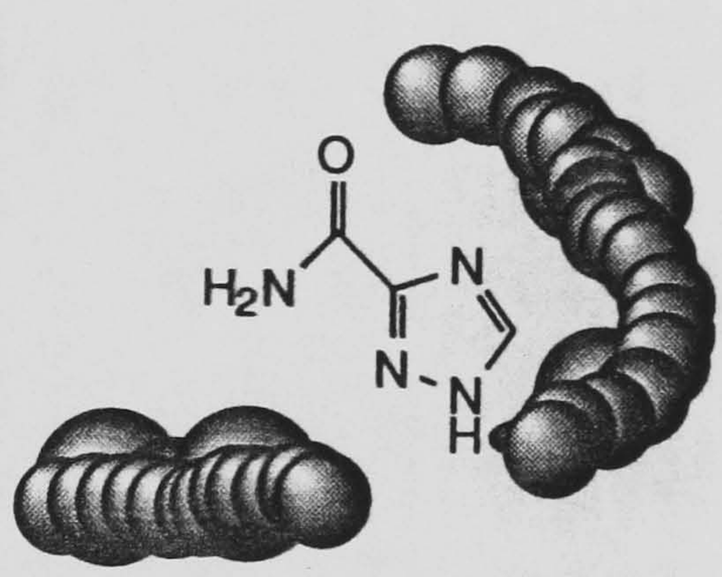


Fig. 3.22 Purines with bulky substituents on position 6 which are acceptors for the *N*-deoxyribosyltransferases from *L. helveticus*

The triazole probably docks in the enzyme site in the same way as the purines do. In this way the bulky substituents on the carboxamide can be accommodated, which roughly corresponds to position 6 on the purine base (Fig. 3.23).



99

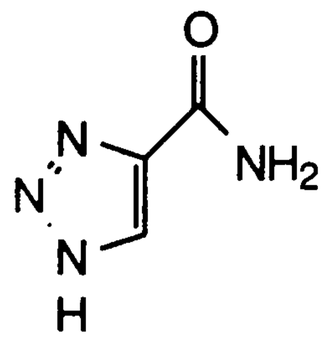
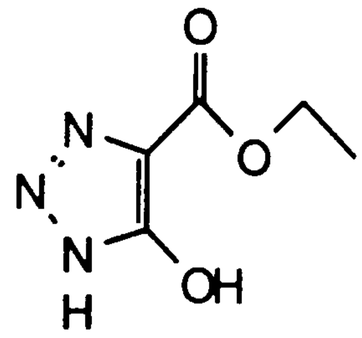
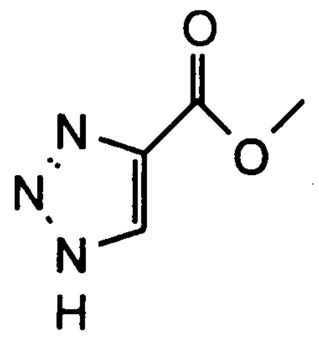
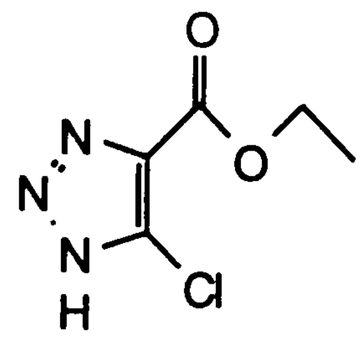
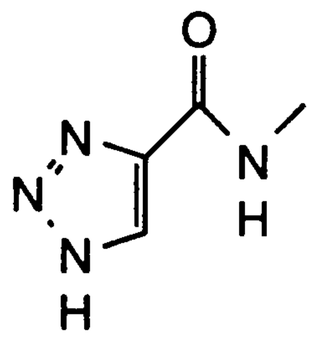
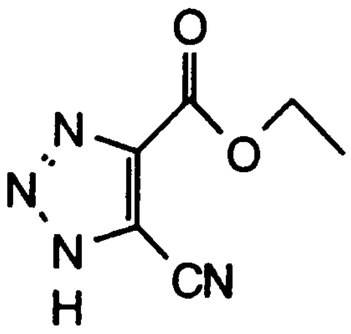
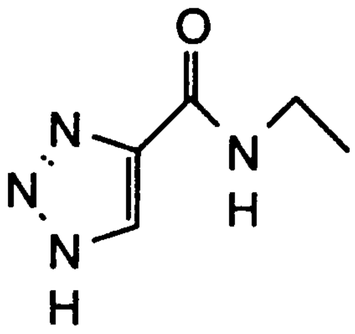
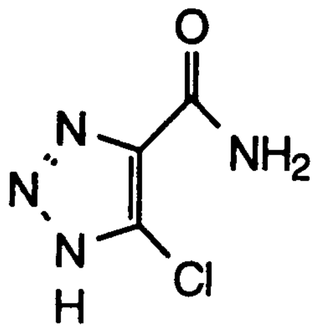
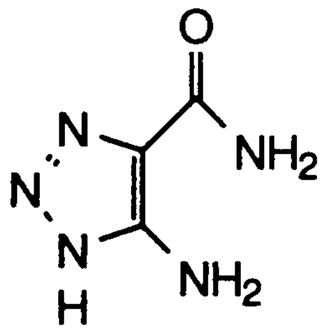
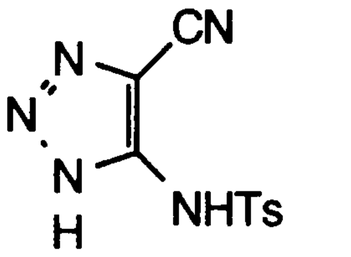


18

Fig. 3.23 Possible position of the triazole base within the active site of the enzyme

1,2,3-Triazole Bases

The pattern of transfer with the 1,2,3-triazoles is not so straightforward. The only bases that were substrates for the *N*-deoxyribosyltransferase reaction were the 1,2,3-triazole-4-carboxamide **30** and the 5-amino-1,2,3-triazole-4-carboxamide **60**. More notable is that the *N*-methyl-1,2,3-triazole-4-carboxamide **38** and *N*-ethyl-1,2,3-triazole-4-carboxamide **39** (Table 3.6) do not act as acceptors for the enzyme, whereas the equivalent 1,2,4-triazole-3-carboxamides were substrates (Table 3.5).

30		✓	55		✗
35		✗	59		✗
38		✗	56		✗
39		✗	58		✗
60		✓	48		✗

Ts = $CH_3OC_6H_4SO_2$-

✓ is an acceptor in the *N*-deoxyribosyltransferase reaction

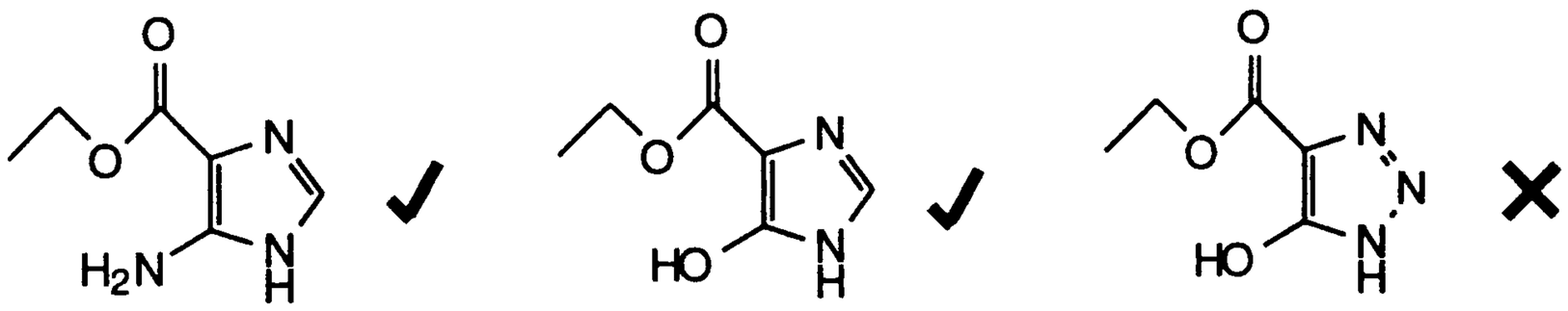
✗ is not an acceptor in the *N*-deoxyribosyltransferase reaction

Table 3.6 Results with 1,2,3-triazole bases used as acceptors in the *N*-deoxyribosyltransferase reaction

Clearly, the enzyme can differentiate between the 1,2,3-triazole and the 1,2,4-triazole bases. The positions of the nitrogen atoms within the 1,2,3-triazole ring must hinder its approach to the active site. The 1,2,3-triazole base could bind in such a way in the active site as to make it incapable of

reacting with the glycosyl-enzyme intermediate. This could be due to a mixture of hydrogen bonding and hydrophobic interactions within the active site.

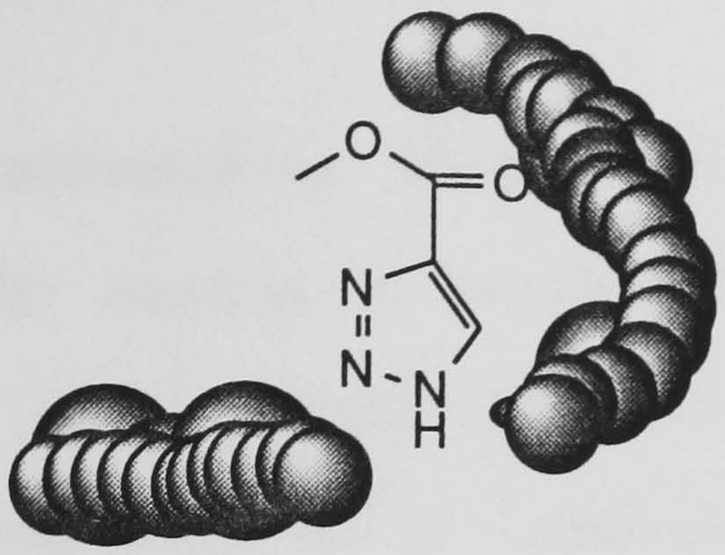
The 1,2,3-triazole esters **35**, **55**, **56** and **59** do not transfer (Table 3.6). This is consistent with the results obtained for the 1,2,4-triazole bases (Table 3.5). However, ethyl-5-amino-imidazole-4-carboxylate **128** and ethyl-5-hydroxyl-imidazole-4-carboxylate **127** have been shown to be acceptors for *N*-deoxyribosyltransferase from *Lactobacillus leichmannii* (Fig. 3.24).[98] How can these facts be reconciled? The 1,2,3-triazole and the imidazole bases only differ in the presence of a nitrogen at position 2, corresponding to position 8 on the purine ring (Fig. 3.24).



128 **127** **55**

Fig. 3.24 Comparison of the structures of the 1,2,3-triazole and imidazole bases

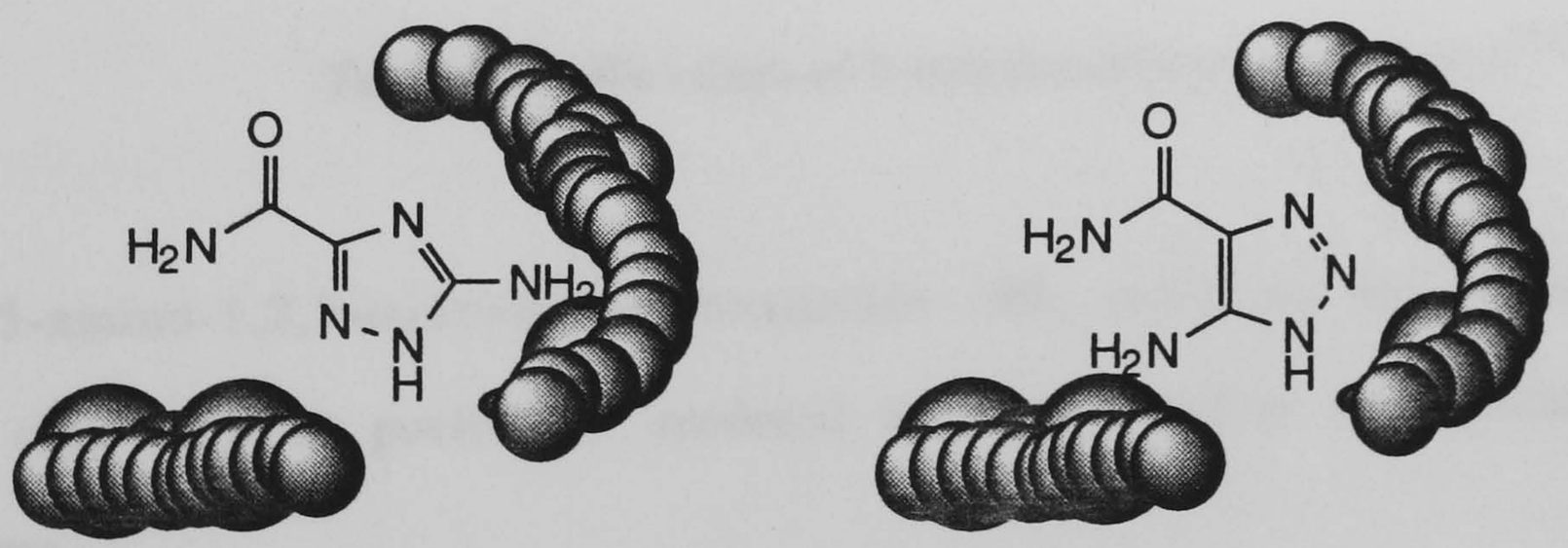
As previously mentioned, purine bases substituted at position 8 act as acceptors but at much slower rates. The presence of a nitrogen at position 8 results in lower reaction velocities. Thus it might be favourable for the 4-substituted-1,2,3-triazole base to bind as shown in (Fig. 3.25), where N-2 is not close to the active site.



55

Fig. 3.25 An alternative way for the 1,2,3-triazole to bind in the enzyme active site

However, in this position the base cannot act as a substrate for the enzyme. With the 1,2,3-triazole-4-carboxamide **30** and the 5-amino-1,2,3-triazole-4-carboxamide **60**, the only bases that acted as acceptors, it appears that the amide protons are crucial. The strong hydrogen bonding of the amide protons might overcome the tendency of the 1,2,3-triazole to minimise the interactions between N-2 and the active site. The 5-amino-1,2,3-triazole-4-carboxamide **60** could then dock in the same way in the active site as 5-amino-1,2,4-triazole-3-carboxamide **13** (Fig. 3.26).



13 **60**

Fig. 3.26 Model of 1,2,4-triazole and 1,2,3-triazole bases in the enzyme active site

Thus, with the *N*-substituted-1,2,3-triazole-4-carboxamides lacking an hydrogen, the 1,2,3-triazole base reverts back to the inactive way of

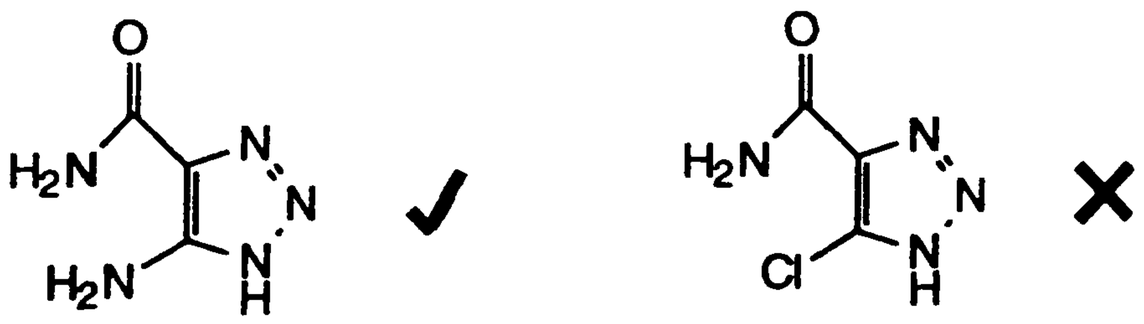
binding.

Another factor is the nucleophilicity of the base. The base acts as a nucleophile in the transfer reaction, a reduction in nucleophilicity could render the base inactive as a substrate. Imidazole has a pKa of 14.44 compared with 1,2,3-triazole which has a significantly lower pKa of 9.26 (Table 3.15). The presence of the third nitrogen in the ring pulls electron density from the ring causing a marked decrease in basicity. Equating basicity with nucleophilicity, imidazole is a much better nucleophile than 1,2,3-triazole and thus would be a better acceptor in the transferase reaction. Esters are electron acceptors and reduce the basicity of the triazole even more making the triazole ester **55** inactive compared to the imidazole ester **127**.

Heterocyclic base	Basic pKa
Imidazole	6.95
1,2,3-Triazole	1.17
1,2,4-Triazole	2.20

Table 3.15 pKa values of 5-membered heterocyclic bases[218]

With 5-amino-1,2,3-triazole-4-carboxamide **60**, replacing the amino group with a chloro **58** at position 5 rendered the base inactive as a substrate for the enzyme.



60 **58**

Fig. 3.25 Comparison of 5-substituted-1,2,3-triazole-4-carboxamides

The amino group is an electron resonance donor and thus base strengthening whereas the halogens are inductive acceptors and hence decrease the basicity of the triazole. The chloro triazole **58** could be too weak a nucleophile to react with the enzyme. Another reason could be that steric factors might hinder the base from binding to the active site.

Conclusion

It is difficult to formulate an overall theory encompassing the results of the purine, imidazole and triazole bases. A combination of steric, hydrogen bonding and electronic factors have been used to explain most of the observations. The triazole bases synthesised are small enough to fit in the active site. However, it is clear that the *N*-deoxyribosyltransferases are sensitive to changes within the imidazole moiety of the ring. It would be useful to compare these findings with the X-ray crystal structure of a recombinant *Lactobacillus leichmannii* nucleoside deoxyribosyltransferase which has been reported. Unfortunately, only the preliminary findings have been published[100] and it is unclear whether this enzyme is like *N*-deoxyribosyltransferase I or *N*-deoxyribosyltransferase II.

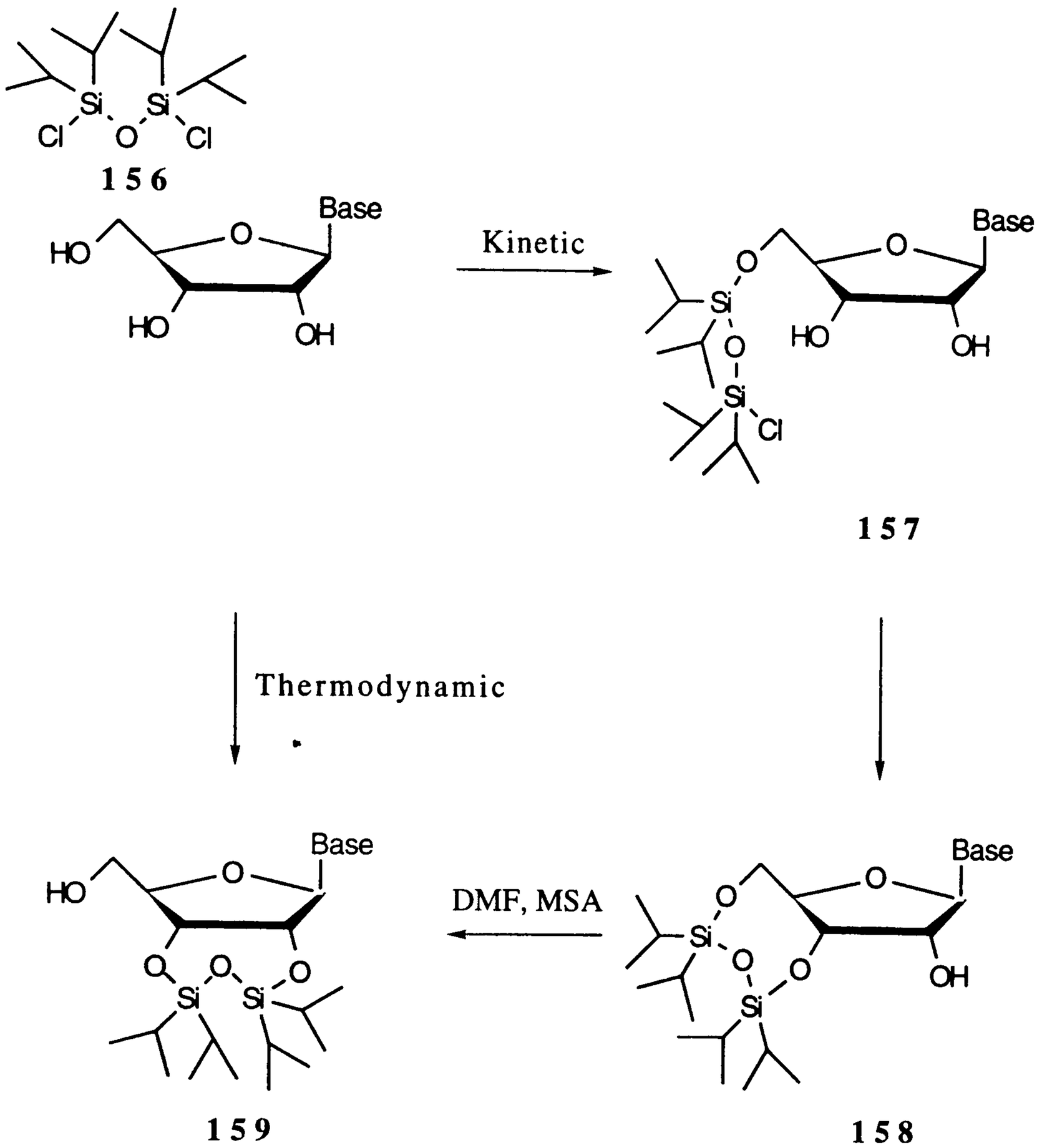
CHAPTER 4

CHEMICAL SYNTHESIS OF NUCLEOSIDE ANALOGUES

The 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (TDPS) Protection Group

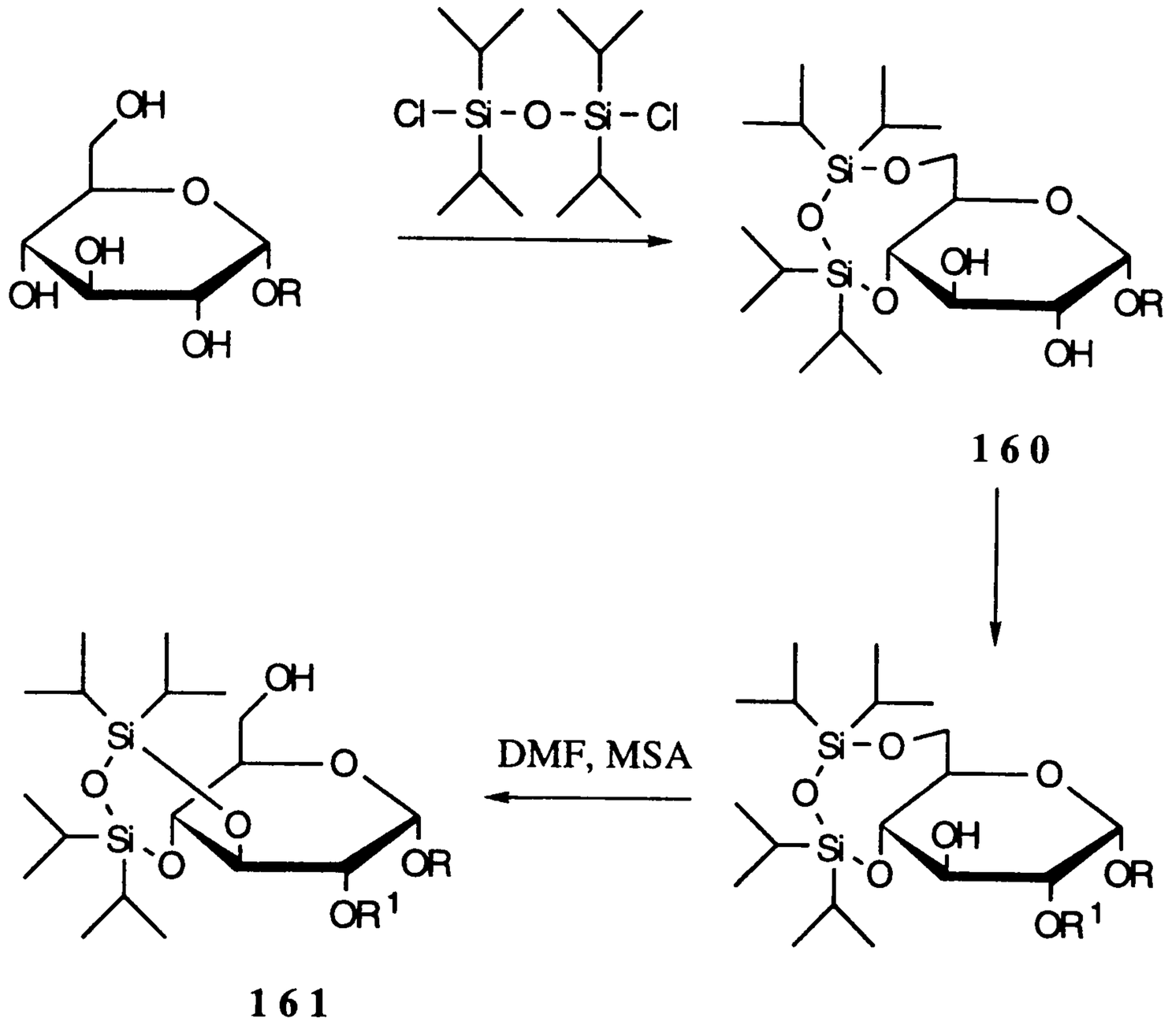
There are many procedures for the modification of the sugar moiety of the intact nucleoside.[47, 219] The regiospecific deoxygenation of ribonucleosides had been impeded by the difficulty in differentiating 2'- and 3'-hydroxyl groups. This problem was solved by Markiewicz, who developed the selective protection of the 3'-hydroxyl and 5'-hydroxyl functions of nucleosides by 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane **156** (Scheme 4.1).[220] 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane **156** reacts rapidly with the primary hydroxyl group on C-5' **157**. This is followed by the slower intramolecular ring closure with the secondary 3'-hydroxyl, to give the 3'-5'-*O*-protected nucleoside **158** (Scheme 4.1).

The tetraisopropyldisiloxane (TPDS) group is stable under a wide range of conditions and so has been extensively used in the 2'-hydroxyl modification of nucleosides.[210] The 3'- and the 5'- ends can be cleaved selectively under appropriate conditions.[220] However, isomerisation is known to occur in acid where the protected nucleoside undergoes 5'-end cleavage to give the 2'-3'-*O*-TPDS derivative **159** (Scheme 4.1).[221] The reaction only occurs in dimethylformamide (DMF) with mesitylene sulfonic acid (MSA) as catalyst.



Scheme 4.1 Reaction of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane with a ribonucleoside

Other solvents, such as, dioxane, acetonitrile, tetrahydrofuran or chloroform are not effective. This flexible property of the TPDS group has been exploited in the synthesis of carbohydrates where the TPDS group was used first to protect the primary and secondary alcohols **160** (Scheme 4.2). Later on in the synthesis, using acid catalysis, the TPDS group was redirected to protect two secondary alcohols **161**.[222]



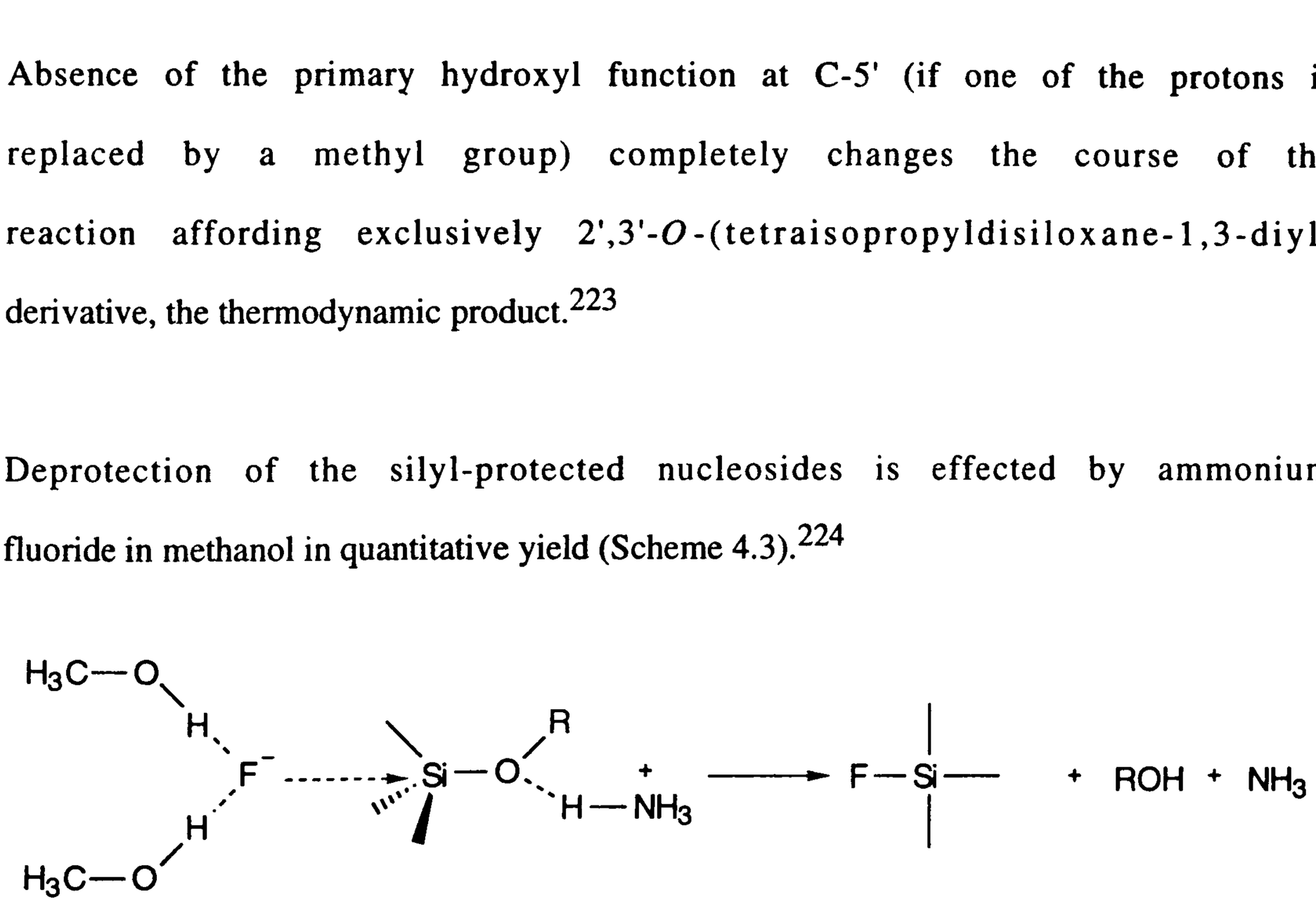
160

161

Scheme 4.2 Manipulation of the TPDS protection group[222]

Absence of the primary hydroxyl function at C-5' (if one of the protons is replaced by a methyl group) completely changes the course of the reaction affording exclusively 2',3'-*O*-(tetraisopropyldisiloxane-1,3-diyl) derivative, the thermodynamic product.[223]

Deprotection of the silyl-protected nucleosides is effected by ammonium fluoride in methanol in quantitative yield (Scheme 4.3).[224]

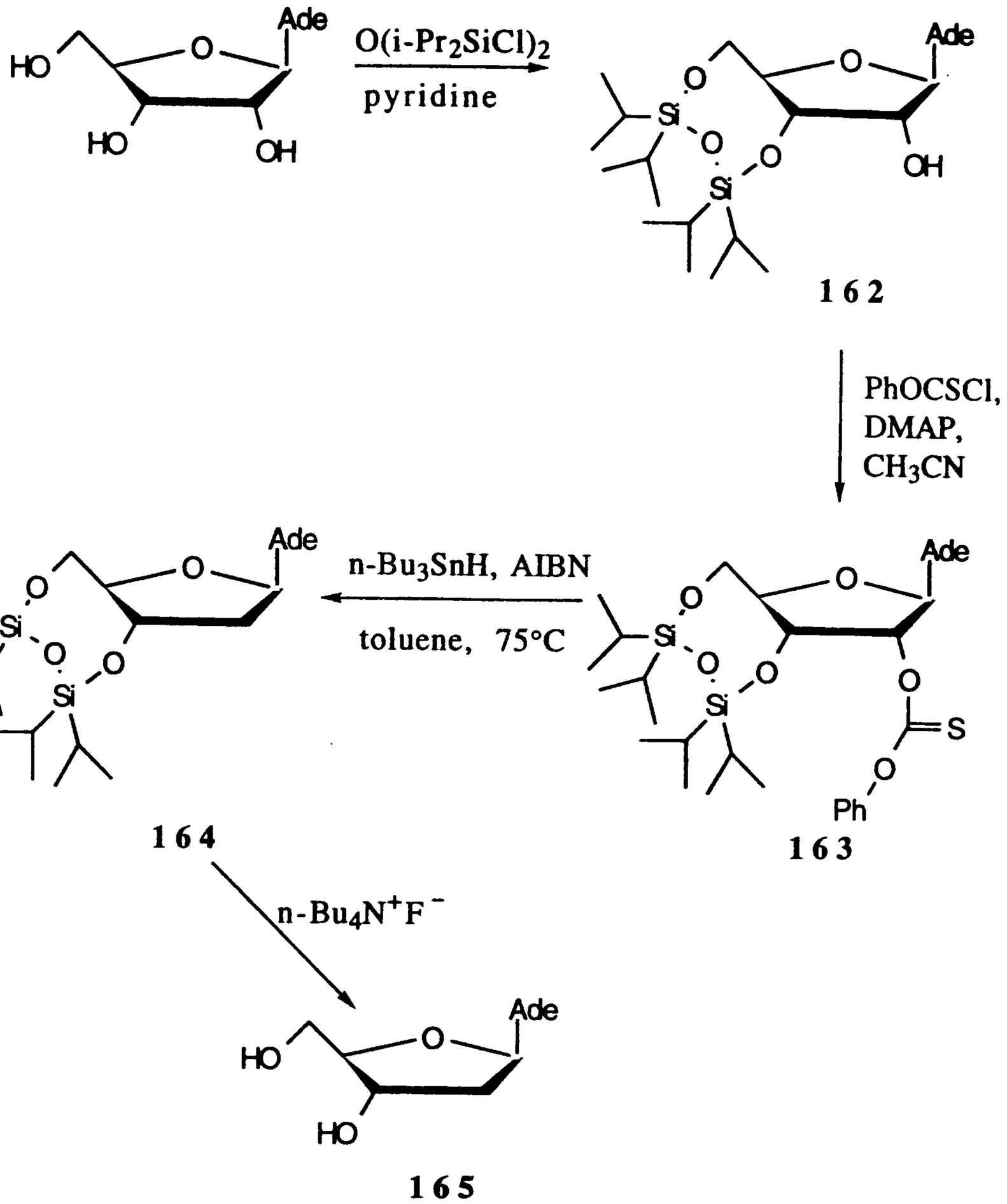


Scheme 4.3 Deprotection of silyl ethers with ammonium fluoride[224]

Ammonium fluoride provides an economical, effective alternative to tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) which until recently, has been the most common way to deprotect silyl ethers. The phase transfer properties of the tetrabutylammonium cation often cause difficulties in the workup and purification of products.

Synthesis of 2'-Deoxyribosylnucleosides

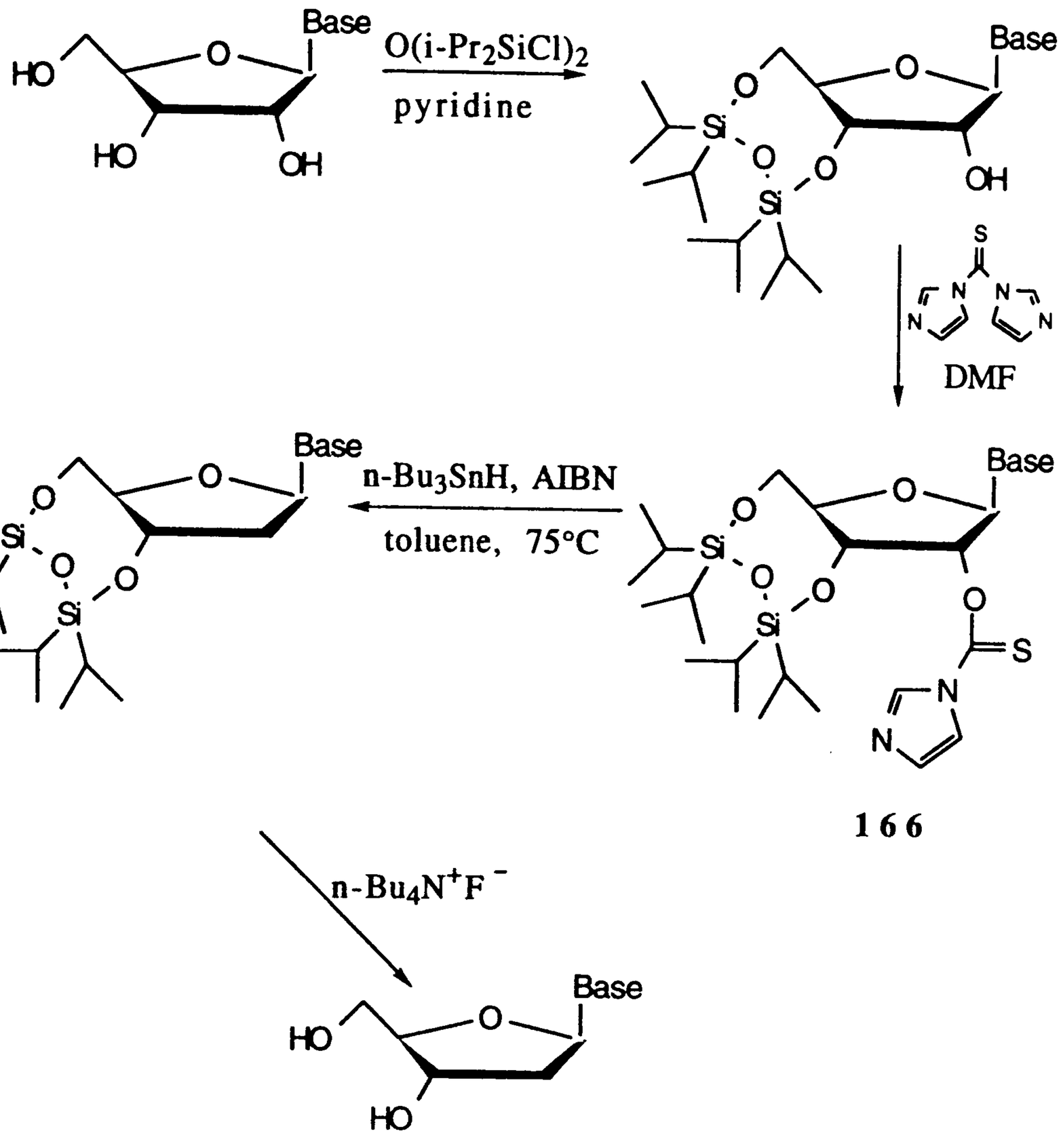
Robins *et al* were the first to report a general four step regiospecific stereoselective procedure for the conversion of ribonucleosides to 2'-deoxyribonucleosides (Scheme 4.4).[225, 226] The first step incorporates the Markiewicz protection of the ribonucleoside as its 3',5'-*O*-TPDS derivative **162**, phenoxythiocarbonylation of its 2'-hydroxyl group to give the xanthate ester **163**, deoxygenation **164** and deprotection using TBAF to give the 2'-deoxynucleoside **165** (Scheme 4.4).



162 **163** **164** **165**

Scheme 4.4 The regiospecific and stereoselective conversion of ribonucleosides to 2'-deoxynucleosides[226]

A variant of the procedure by Watanabe and coworkers utilises the deoxygenation of the 2'-*O*-(imidazol-1-yl) thionocarbonyl derivative **166** (Scheme 4.5).[227]



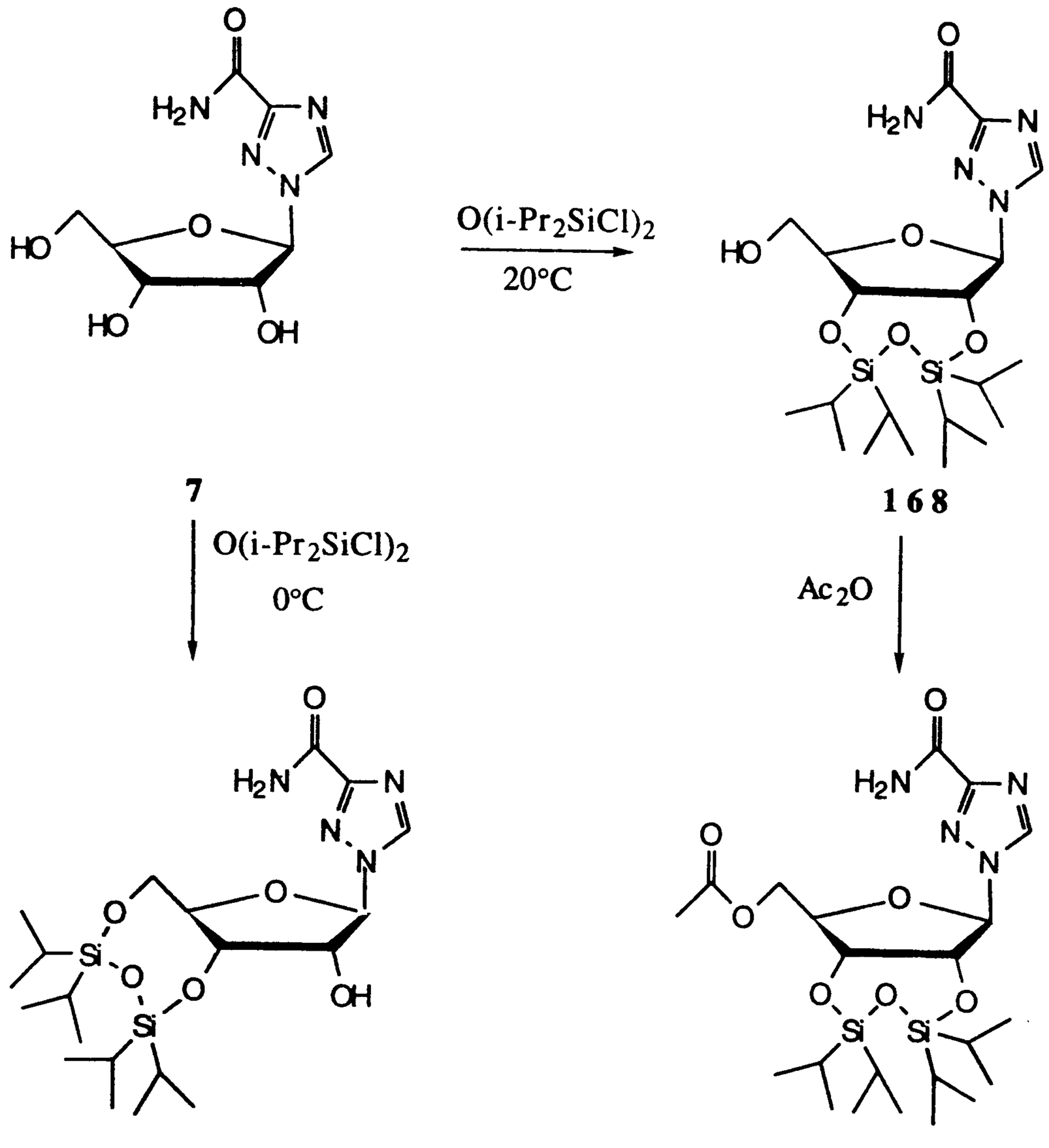
Scheme 4.5 The synthesis of 2'-deoxyribonucleosides *via* the 2'-*O*-{(imidazol-1-yl)thiocarbonyl} nucleoside[227]

Sanghvi *et al*[135] were able to apply this method, using the 3'-*O*-(imidazol-1-yl) thiocarbonyl of 5'-protected-2'-deoxyribavirin, in the synthesis of 2',3'-dideoxyribavirin.

Synthesis of 5'-Deoxyribavirin

The chemical synthesis of 2'-deoxyribavirin was attempted because the 2'-deoxyribavirin, synthesised enzymatically, was found to possess some antiviral activity. The difficulties with the actual purification of 2'-deoxyribavirin in the enzymatic synthesis led to the exploration of

chemical routes to this product. The deoxygenation of ribavirin at C-2' seemed to be an easy procedure with plenty of precedence for this type of transformation. The first step was the selective protection of the the 3',5'-hydroxyl groups of ribavirin as outlined by Robins.[226] Ribavirin **7** and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane **156** in either anhydrous pyridine or DMF and imidazole gave 66% of the protected nucleoside. This was assumed to be the 1-(3',5'-*O*-(tetraisopropyldisilox-1,3-diyl)-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide **167**. However, acetylation of the *O*-TPDS-protected ribavirin **168** showed that the 2'- and 3'-hydroxyl functions of the nucleoside were protected as in compound **169** (Scheme 4.6). The electronwithdrawing effect of the acetyl group causes the protons attached to shift to lower field in the ^{1}H NMR.[228] The chemical shift of the protons at C-5' were 3.56 ppm in the *O*-TPDS-protected ribavirin **168** which shifted to 4.34 ppm **169** on acetylation. Thus the product was 1-(2',3'-*O*-(tetraisopropyldisilox-1,3-diyl)-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide **168**. The chemical shifts of the protons on C-2' and C-3' were unchanged.



Scheme 4.6 Protection of ribavirin with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane

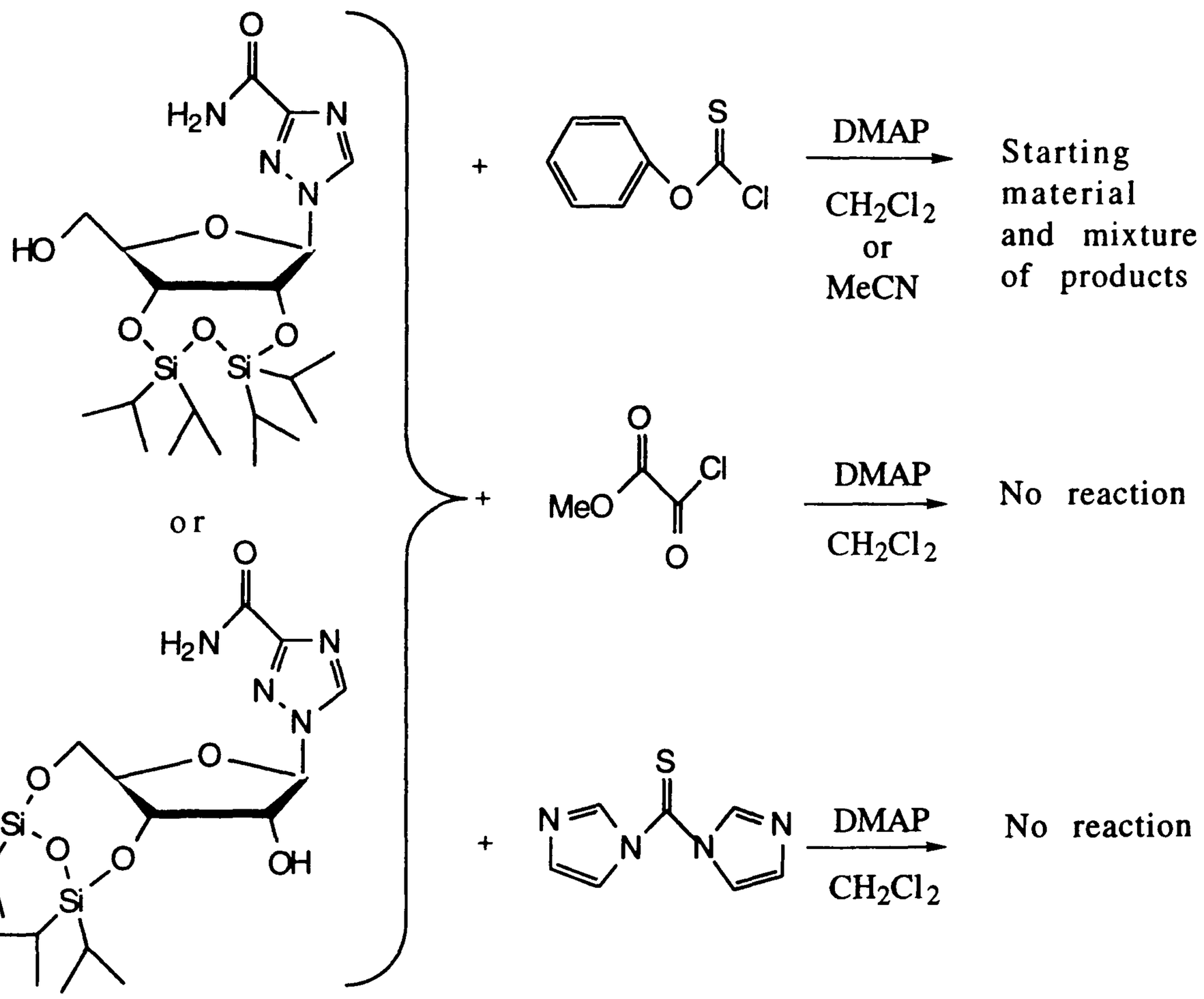
Maintaining the temperature at 0°C in pyridine in the initial stages of the reaction[229] ensured that the kinetic product, 3',5'-*O*-TPDS-ribavirin **167**, and not the thermodynamic 2'-3'-*O*-protected nucleoside **168**, was formed in 41% yield.

Attempts to form the phenyl thiocarbonylxanthate ester[226, 230] of either the 2',3'-*O*-TPDS-ribavirin **168** or 3',5'-*O*-TPDS-ribavirin **167** were not successful (Scheme 4.7). When dry dichloromethane was used as solvent no reaction occurred, whereas reaction in acetonitrile gave a range of

products, the number of which increased with time, but the starting material was always present by TLC.

Reaction of 2',3'-*O*-TPDS-ribavirin **168** and 3',5'-*O*-TPDS-ribavirin **167** with methyloxalyl chloride in dichloromethane gave no reaction by TLC and only starting material was isolated in the workup (Scheme 4.7).

Formation of the 2'-*O*-(imidazol-1-yl) thiocarbonyl of 3',5'-*O*-TPDS-ribavirin in dichloromethane[231] was not successful (Scheme 4.7). No reaction seemed to occur by TLC.

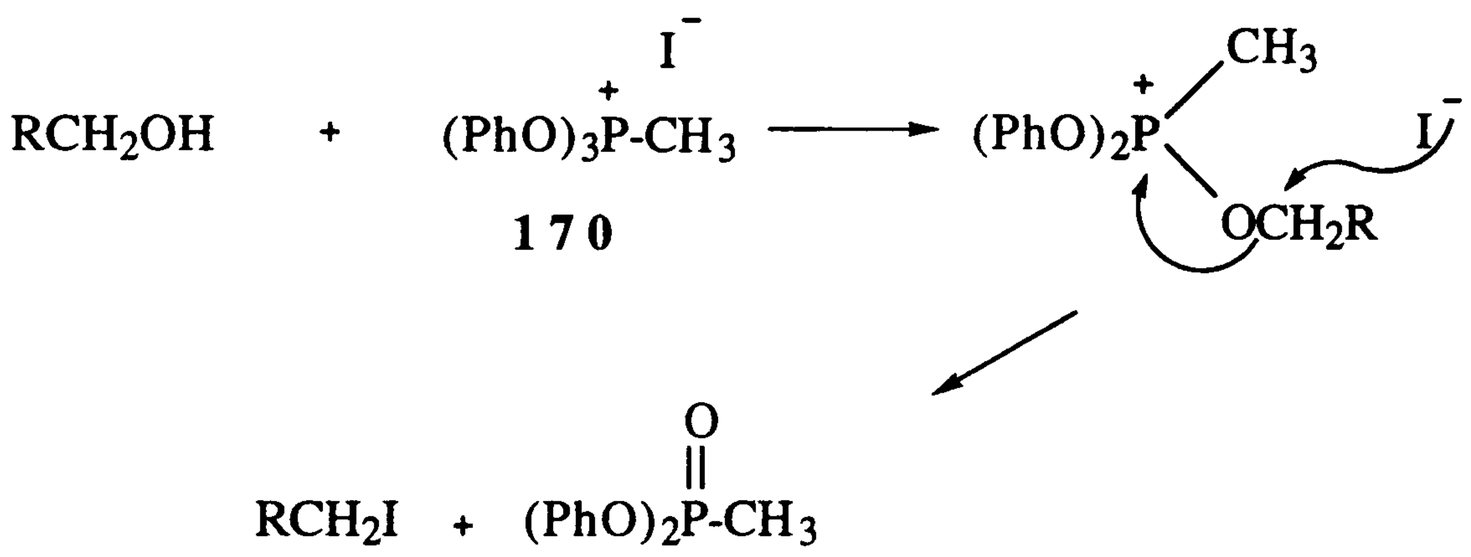


Scheme 4.7 Attempts at the modification of the free hydroxyl group of TPDS protected ribavirin

The successful synthesis of 2',3'-dideoxyribavirin from 2'-deoxyribavirin

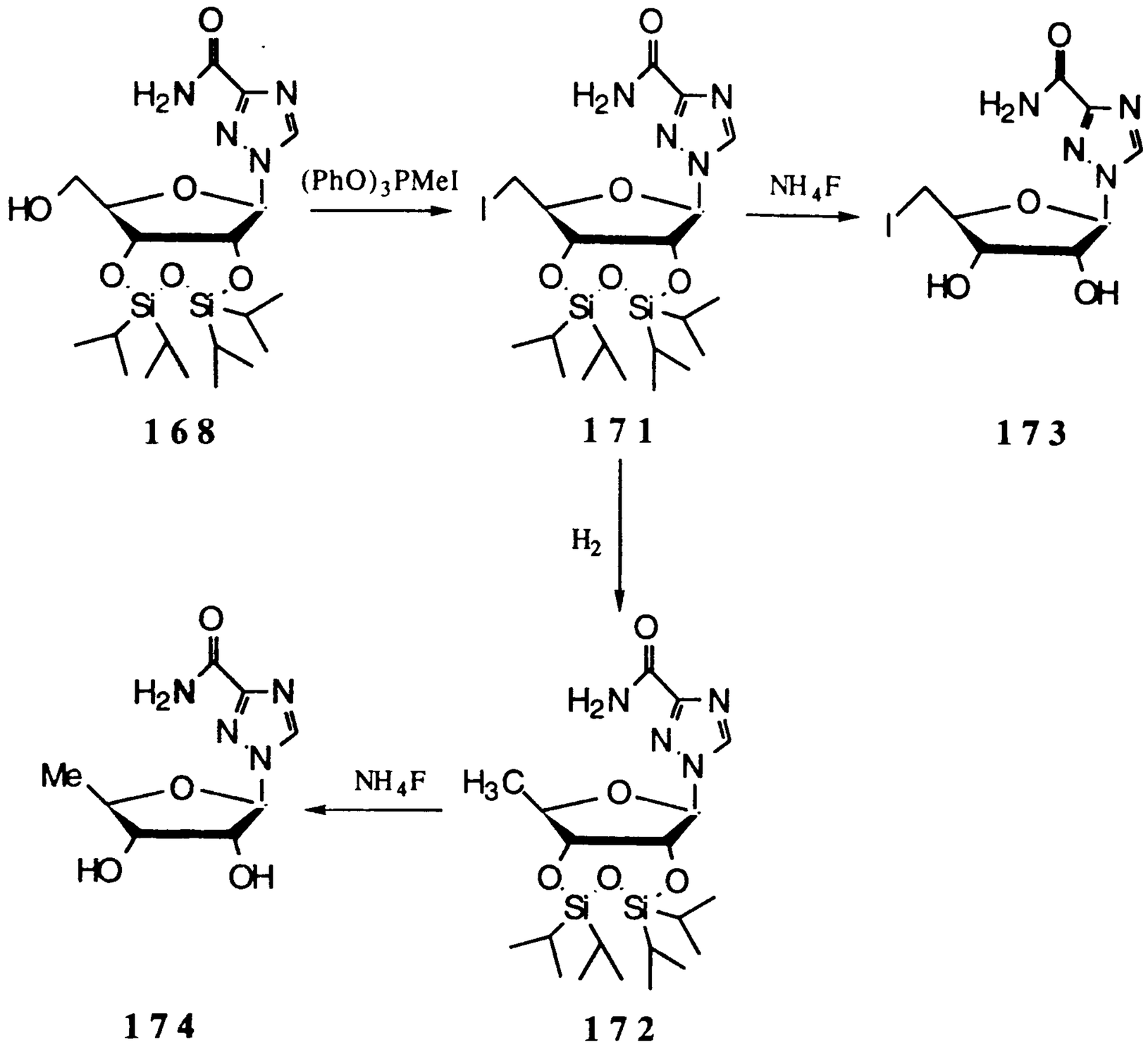
using an excess of *N*,*N*''-thiocarbonyldiimidazole in DMF, followed by reductive deoxygenation has been reported.[135] It is difficult to explain the lack of success with this reagent. The use of dichloromethane instead of DMF and possible steric factors could account for this.

Another approach was tried. A wide range of 2'-deoxynucleosides has been prepared *via* either reduction of 2'-halogenated compounds or desulfurisation of various thio derivatives.[47] A procedure by Verheyden *et al*[232] was used to iodinate the remaining hydroxyl group of 2',3'-*O*-TPDS-ribavirin **168** with methyltriphenoxyphosphonium iodide (Rydon's reagent) **170** in anhydrous DMF. It is thought to react with alcohols in the following way (Scheme 4.8).



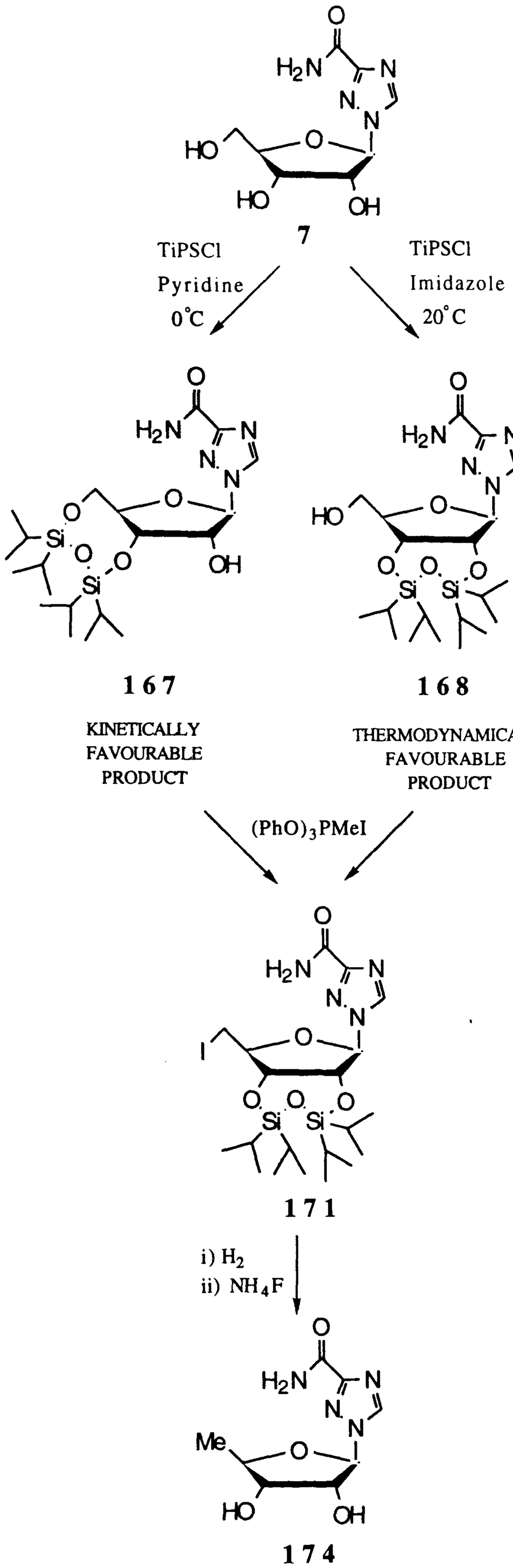
Scheme 4.8 Iodination of the hydroxyl group using Rydon's reagent[47, 232]

This gave the expected product **171** in 62% yield (Scheme 4.9). The palladium-catalysed reduction[148] of the iodo protected nucleoside gave the 5'-deoxy-2',3'-*O*-TPDS-ribavirin **172** in 63% yield. Deprotection with ammonium fluoride in methanol resulted in 5'-iodo-5'-deoxyribavirin **173** and 5'-deoxyribavirin **174** in yields of 91.5% and 84% respectively.



Scheme 4.9 Synthesis of 5'-iodo-5'-deoxyribavirin and 5'-deoxyribavirin from 2',3'-*O*-TPDS-ribavirin

Iodination, followed by reduction of 3',5'-*O*-TPDS-ribavirin **167**, in exactly the same procedure as detailed above, gave after work up, the 5'-deoxyribavirin **174** (Scheme 4.10). Further investigations showed that the iodo-protected derivative was the 5'-iodo-5'-deoxy-2',3'-*O*-TPDS-ribavirin **171**, identical, by ^{1}H NMR and TLC, with that previously made from 3',5'-*O*-TPDS-ribavirin **168**. Iodination caused isomerisation of the TPDS group from the 3'- and 5'- position to the 2'- and 3'- position and the only product of this reaction was the 5'-iodo-5'-deoxy-2',3'-*O*-TPDS-ribavirin **171** (Scheme 4.10).



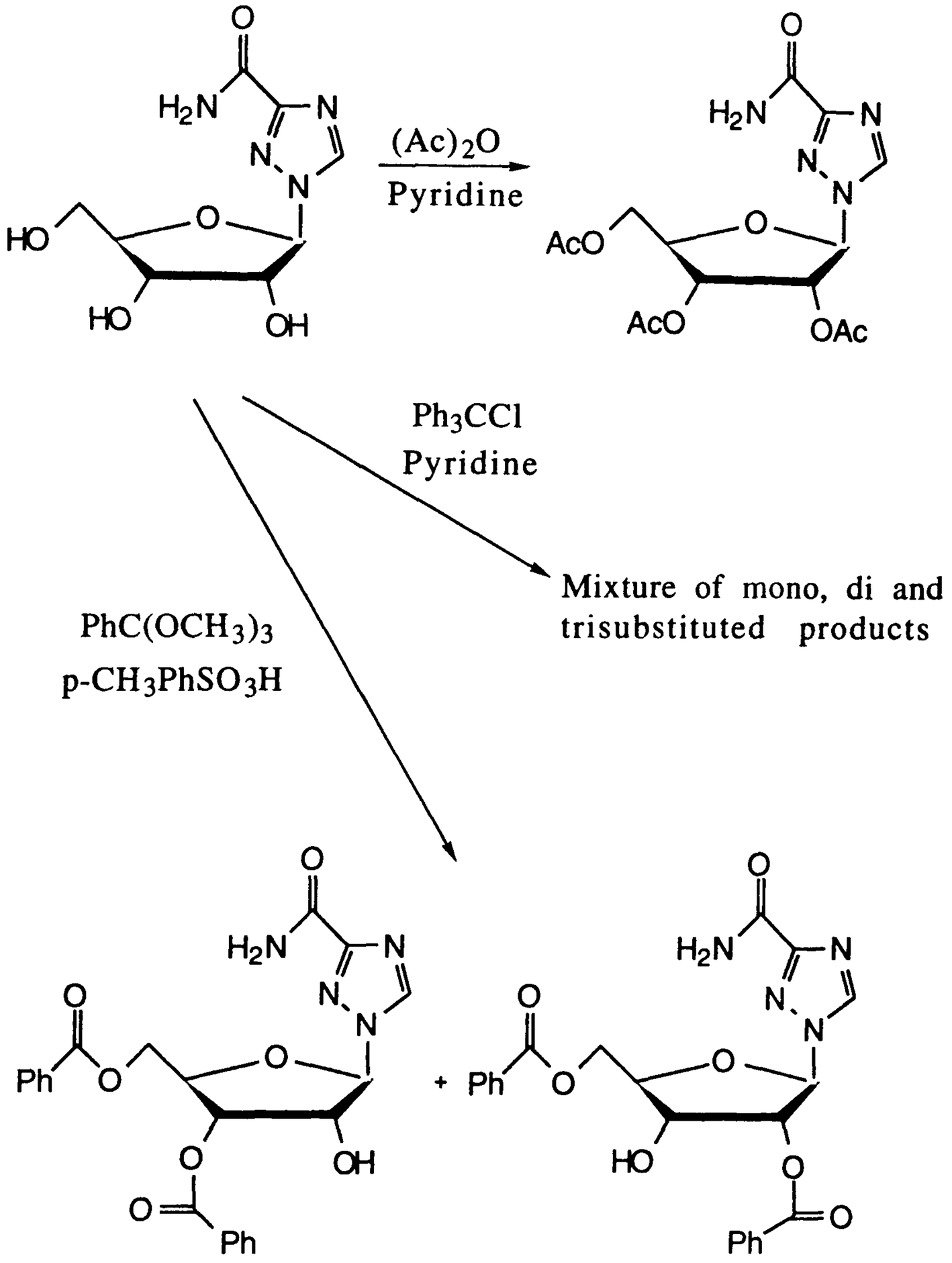
Scheme 4.10 Synthesis of 5'-deoxyribavirin

Clearly, the difference in stability between 3',5'-*O*-TPDS-ribavirin **167** and 2',3'-*O*-TPDS-ribavirin **168** is far greater than in other TPDS protected nucleosides. At room temperature, the protection of ribavirin by 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane is governed by thermodynamic rather than kinetic factors. Conducting the reaction at 0°C gives only the kinetic product, which is stable indefinitely in the solid form. Difficulties have been reported in the synthesis of 2'- and 3'- derivatives of ribavirin.[229] After the synthesis of the 3',5'-*O*-TPDS-ribavirin **167**, careful manipulation of the reaction conditions was required to give the desired 2'-ester. The removal of the protective disiloxyl group gave a range of products. Finally, the desired products were synthesised by the non-selective acylation of ribavirin followed by chromatographic separation and purification.

Another protection procedure has been reported in the synthesis of a range of deoxygenated ribavirin derivatives.[134] Acylation of ribavirin with α-acetoxyisobutyryl bromide gave a mixture of intermediate isomers, which were not characterised. On reduction only the 3'-deoxyribavirin was isolated.

Other protective groups were tried (Scheme 4.11). Protection with the trityl group[233] gave a complex mixture of products. Three were partially isolated by flash chromatography but not properly identified. A test reaction was followed by TLC over time to see the order in which the products emerged. The aim was to optimise the product ratio by carefully picking the time when the reaction was quenched. Unfortunately, what appeared to be the monoprotected ribavirin- as it was the first product to appear in the reaction, was still present when the third spot appeared- that is the triprotected ribavirin. The products were difficult to separate as the Rfs were very close. This procedure was abandoned as it was too wasteful.

Protection with trimethylorthobenzoate gave a mixture of products in 3:1 ratio (20.2% : 7.6%) of the 2',5'-*O*-phenylesters and 3',5'-*O*-phenylesters (Scheme 4.11).[234] The two products, having identical Rf values, could not be separated by flash chromatography.



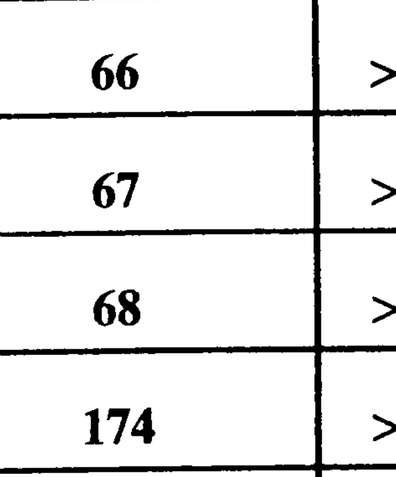
Scheme 4.11 Protection of the hydroxyl groups of ribavirin

The synthesis of 2'-deoxyribavirin was repeated using thymidine and *N*-deoxyribosyltransferase, which, though it presented difficulties in purification, was less troublesome than the chemical procedures. This is a classic example of the advantages of the enzymatic over purely chemical methods.

Results of Viral Assays

Nucleoside	HCMV /μM	HSV-2 /μM	VZV /μM	Flu A /μM	HIV /μM	Flu B /μM	$CCIC_{50}$ Vero /μM
7	-	-	-	30	-	13	
65	49	>10	>40	3.3	-	IC50= 1.9μM	-
66	>100	>100	>40	>100	-	-	-
67	>100	>100	>40	>100	>50	-	>500
68	>100	>100	>100	>100	>50	-	>500
174	>100	>100	>100	>100	ST50	-	>500
173	>10 <100	ST10	>40	>10 <100	>50	-	196

Table 4.1 The EC_{50} values of some of the nucleosides synthesised



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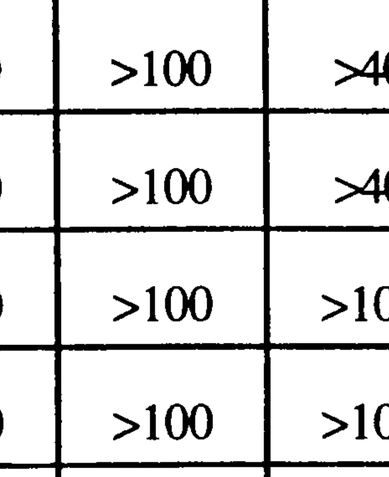

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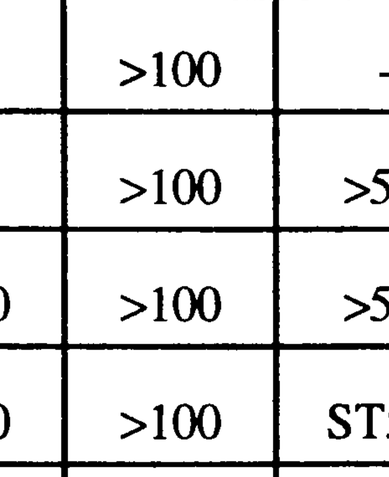

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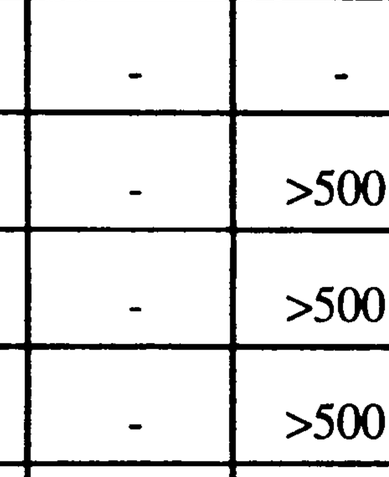

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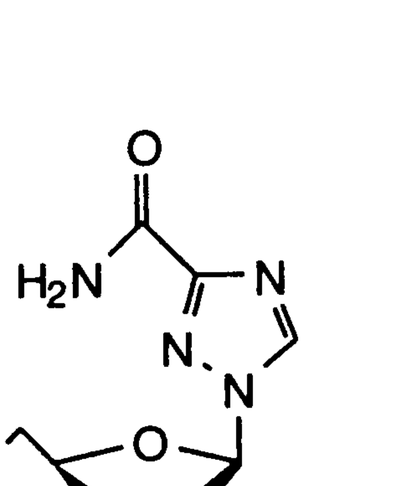

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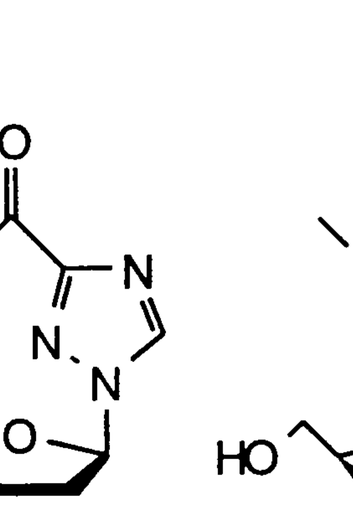
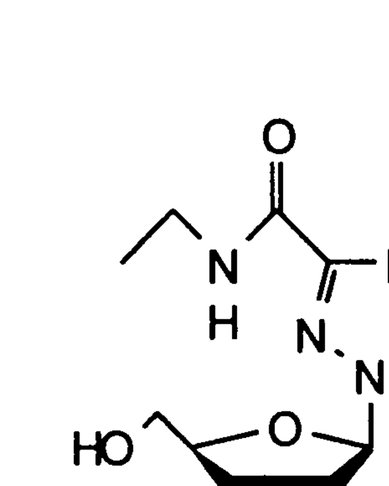


173



174

Fig. 4.1 Nucleoside analogues tested for antiviral activity

EC_{50} represents the concentration of the compound that inhibits the virus in infected cells to 50% of the control. $CCIC_{50}$ Vero is the measure of the cell toxicity, representing the concentration of the nucleoside that kills 50% of the cells. 2'-Deoxyribavirin **65** showed significant antiviral activity. This is in contrast to findings by Sidwell *et al*, who reported that the 2'-deoxyribofuranosyl analogue was inactive against both RNA and DNA viruses.[43] Compared to ribavirin **7** the remainder of the nucleosides synthesised showed little antiviral activity (Table 4.1). Alkyl substitution of the carboxamide group renders the 2'-deoxyribavirin analogues inactive.

CHAPTER 5

GENERAL MATERIALS AND METHODS

Materials

Chemicals and starting materials were either commercially available or synthesised as described in the text. Chemicals were either purified using literature methods[235] or purchased as the highest available grade. All solvents were dried and distilled before use.

Methods

^{1}H NMR spectra were recorded at 250 MHz on a Bruker ACF250 spectrometer or at 400 MHz on a Bruker WH400 spectrometer. Chemical shifts are given in ppm relative to tetramethlysilane (TMS) (0.00ppm). Multiplicities of ^{1}H NMR signals, where applicable, are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), ddd (doublet of doublet of doublets) and m (multiplet).

^{13}C NMR spectra were recorded at 100.62 MHz on a Bruker WH400 spectrometer or at 62.89 MHz on a Bruker ACF250 spectrometer with $CHCl_3$, $(CH_3)_2SO$ and CH_3OD as internal standards.

Nuclear Overhauser enhancement spectra were measured on a solution that had not been deoxygenated and were acquired with an irradiation time of 2 seconds.

Mass spectra were recorded using a Kratos MS80 instrument. Electron

impact (EI) spectra were recorded at 70 eV and for chemical ionisation (CI) spectra ammonia was used as the carrier gas.

Accurate mass spectra were recorded on either a Kratos MS80 instrument or were carried out by the SERC Mass Spectrometry Service Centre, Swansea.

CHN analysis was carried out at the University of Warwick or The Wellcome Research Laboratories, Beckenham, Kent.

Melting points are uncorrected.

High-Performance Liquid Chromatgraphy (HPLC) analysis was performed on a Waters machine, 501 solvent delivery system. The concentration of nucleosides and bases present in the reaction mixture was determined using reverse phase HPLC on a Techsphere 5C8 or a Techsphere 10ODS column (25 cm x 4.6 mm and a precolumn, 5 cm x 4.6 mm; HPLC Technology Ltd, Macclesfield, Cheshire, UK). The samples were eluted from the column using a mobile phase of acetonitrile and 10 mM ammonium acetate with doubly distilled water at a flow rate of 1.0 ml/min and detected by UV at 214 nm.

TLC analyses were run on aluminium plates coated with silica gel (Merck $60_{F\ 254}$, 0.20 mm) and eluted in the solvent systems given in the text. Visualisation was achieved by UV or by spraying the plate with p-anisaldehyde in ethanol and acetic acid followed by heating to observe the sugars. Compounds were purified by recrystallisation or by flash chromatography on silica gel.

Microorganisms and Media

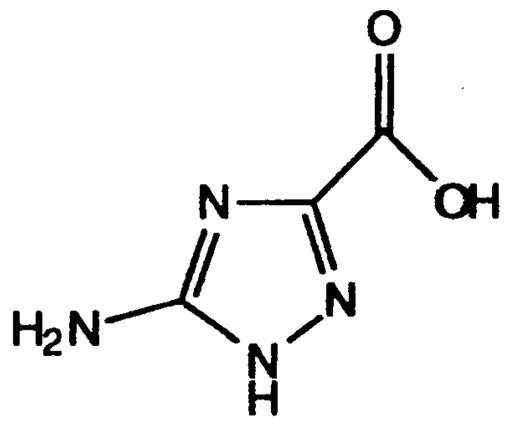
Lactobacillus leichmannii was obtained from the American Type Culture Collection (ATCC, Rochville, Maryland, USA). The growth media MRS Broth[236] was purchased from Oxoid, Basingstoke, Hampshire, UK and was used as instructed on the bottle. The Biorad protein assay was purchased from BioRad, Watford, Hertfordshire, UK.

The symbol * indicates the synthesis of a new compound.

EXPERIMENTAL

Synthesis of 1,2,4-Triazole Bases

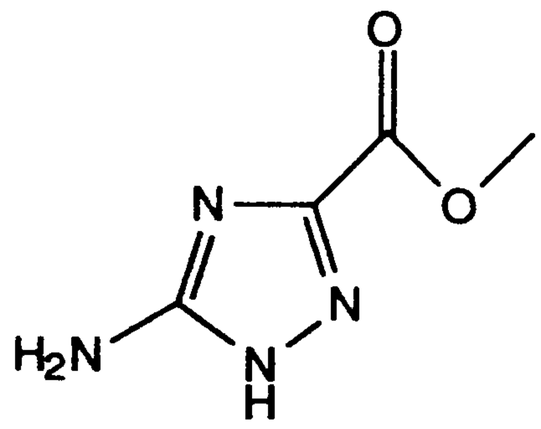
Synthesis of 5-amino-1,2,4-triazole-3-carboxylic acid 10[165, 166]



Oxalic acid (100.8 g, 1.12 mol) was dissolved by heating in water (800 ml). To the cooled solution was slowly added aminoguanidine bicarbonate (95.2 g, 0.68 mol). The mixture was refluxed for 6 h and a precipitate was formed. On cooling, aqueous sodium hydroxide (400 ml, 2.5 M) was added to dissolve the precipitate and the solution was refluxed for 1 h. When cooled the mixture was adjusted to pH 3.0 with concentrated hydrochloric acid. The colourless precipitate was filtered, washed with water and dried to give 5-amino-1,2,4-triazole-3-carboxylic acid **10** (85.0 g, 98%), m.p. 183°C (lit.,[165] 182-3°C); ν_{max} (nujol mull)/cm^{-1} 1684 (C=O); *m/z* (FAB) 128

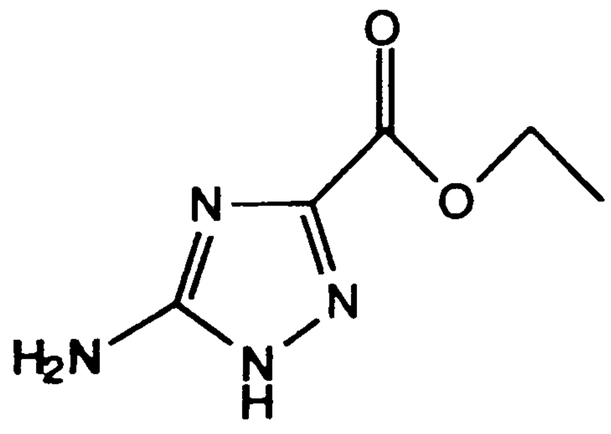
((M+H)$^+$, 7%), 58 (28) and 46 (27).

Synthesis of methyl 5-amino-1,2,4-triazole-3-carboxylate 11[166]



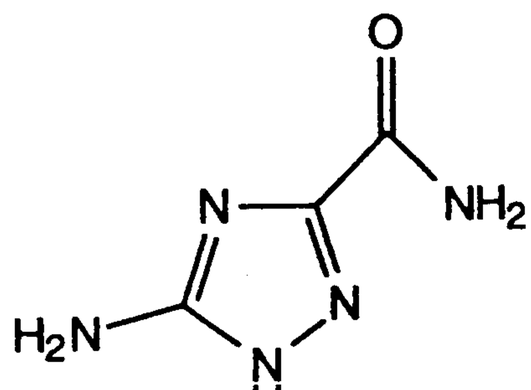
5-Amino-1,2,4-triazole-3-carboxylic acid **10** (5.00 g, 39.0 mmol) was suspended in methanol (50 ml) and saturated with hydrogen chloride gas at 0°C. The solution was left stirring at room temperature for 7 d. Nitrogen gas was bubbled through the solution to remove the excess hydrogen chloride gas. The solution was filtered, the filtrate evaporated to dryness and the residue recrystallised from methanol giving methyl 5-amino-1,2,4-triazole-3-carboxylate **11** (4.345 g, 78.4%) as colourless crystals, m.p. 220°C (lit.,[166] 220°C); ν_{max} (KBr)/cm^{-1} 3371-2982 (C-H), 1707 (C=O) and 1672 (C=O); δ_H (250 MHz; MeOH-d^4) 4.04 (3 H, s, OMe) and 5.17 (1 H, br s, N*H*$_2$); δ_C (250 MHz; MeOH-d^4) 53.96 (OMe), 143.33 (C-5), 153.73 (C-3) and 157.54 (C=O); *m/z* (CI) 160 ((M+NH$_4$)$^+$, 27%), 143 ((M+H)$^+$, 100), 128 (3), 112 (1), 99 (2), 85 (23) and 56 (1); *m/z* (EI) 142 (M$^{+\cdot}$, 27%), 100 (10), 97 (3), 82 (26), 69 (8), 57 (18), 54 (25), 43 (32) and 36 (100).

Synthesis of ethyl 5-amino-1,2,4-triazole-3-carboxylate 12[167]*



5-Amino-1,2,4-triazole-3-carboxylic acid **10** (8.0 g, 62.5 mmol) was suspended in ethanol (150 ml) cooled to 0°C by an ice/water bath and saturated with hydrogen chloride gas for 30 min, then sealed and left stirring at room temperature for 11 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo*, the residue was treated with saturated aqueous sodium hydrogen carbonate (41 ml) and the white precipitate collected, washed with water and dried to give ethyl 5-amino-1,2,4-triazole-3-carboxylate **12** (5.155 g, 53%) as a colourless solid, m.p. 280-283°C (decomp); (Found: C, 38.465; H, 5.165; N, 35.27. $C_5H_8N_4O_2$ requires C, 38.26; H, 5.40; N, 35.02); δ_H (250 MHz; DMSO-d^6) 1.25 (3 H, t, *J* 6.98, Me), 3.51 (2 H, br s, NH_2), 4.22 (2 H, q, *J* 7.17, CH_2) and 12.67 (1 H, br s, H-1); δ_C (250 MHz; DMSO-d^6) 14.15 (Me), 60.43 (CH_2), 151.78 (C-5), 157.63 (C-3) and 160.33 (C=O); *m/z* (CI) 174 ($(M+NH_4)^+$, 7%), 157 ($(M+H)^+$, 30), 96 (100) and 79 (91); *m/z* (EI) 156 ($M^{+\cdot}$, 20%), 128 (9), 78 (100) and 45 (57).

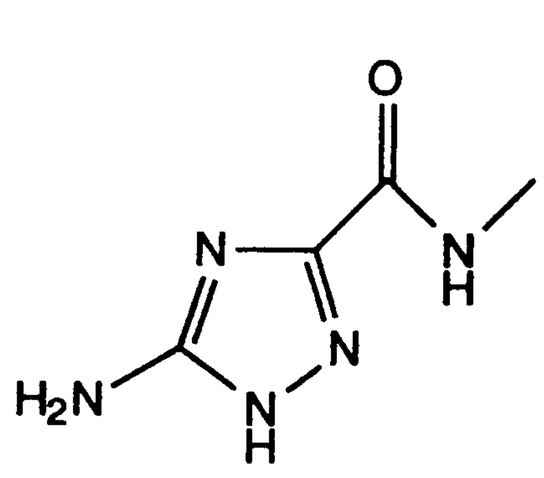
Synthesis of 5-amino-1,2,4-triazole-3-carboxamide 13



Methyl 5-amino-1,2,4-triazole-3-carboxylate **11** (0.66 g, 4.65 mmol) was dissolved in methanol (175 ml), cooled to 0°C and saturated with ammonia gas. The suspension was left stirring at room temperature for 8 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8

column, 25 cm x 4.6 mm, elution with 3% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from water to give 5-amino-1,2,4-triazole-3-carboxamide **13** (0.53 g, 90%) as a colourless crystalline solid, m.p. 295-298°C (decomp); (Found: C, 28.04; H, 3.98; N, 54.83. $C_3H_5N_5O$ requires C, 28.35; H, 3.98; N, 55.09); ν_{max} (KBr disc)/cm^{-1} 3342, 3352, (NH-amide) and 1707, 1691, 1655 (C=O); δ_H (250 MHz; DMSO-d^6) 3.49 (2 H, br s, NH_2/$H_2$0), 6.14 (1 H, br s, NH-amide), 7.44 (1 H, br s, NH-amide) and 12.52 (1 H, s, H-1); δ_C (250 MHz; DMSO-d^6) 154.51 (C-5), 157.33 (C-3) and 161.75 (C=O); *m/z* (CI) 128 ($(M+H)^+$, 45%), 111 (25), 85 (7), 69 (2), 56 (18), 45 (13), 41 (100) and 33 (13); *m/z* (EI) 127 ($M^{+\cdot}$, 47%), 107 (18), 77 (34), 56 (100), 44 (93) and 39 (20).

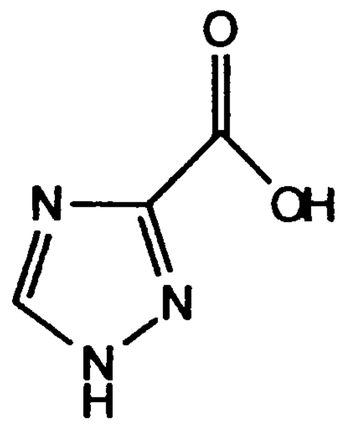
Synthesis of *N*-methyl-5-amino-1,2,4-triazole-3-carboxamide 14[166]*



Ethyl 5-amino-1,2,4-triazole-3-carboxylate **12** (0.224 g, 1.56 mmol) was dissolved in methylamine (20 ml, 25-30% w/v solution in water) and the solution was left at room temperature for 4 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 4% acetonitrile/10 mM ammonium acetate). The solvent was evaporated *in vacuo* and the residue gave on recrystallisation from water *N*-methyl-5-amino-1,2,4-triazole-3-carboxamide **14** (0.197 g, 97.5%) as colourless crystals, m.p. 153-155°C; (Found: C, 34.32; H, 4.95; N, 49.99. $C_4H_7N_5O$ requires C, 34.05; H, 5.00; N, 49.61); δ_H (250 MHz; D_2O) 2.53 (1.5 H, s,

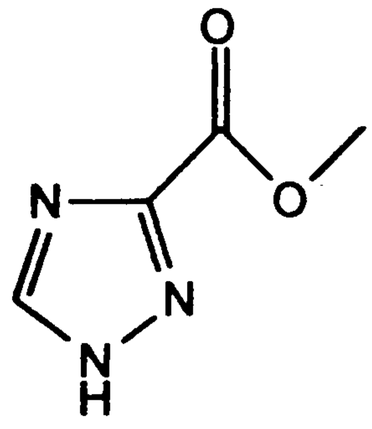
Me) and 2.83 (1.5 H, s, Me); δ_C (250 MHz; D_2O) 25.36 (Me), 26.55 (Me), 154.41 (C-5), 158.15 (C-3) and 162.61 (C=O); *m/z* (CI) 159 ($(M+NH_4)^+$, 40%), 142 ($(M+H)^+$, 100), 112 (2), 102 (2) and 85 (13); *m/z* (EI) 141 ($M^{+\cdot}$, 38%), 112 (13), 98 (8), 84 (20), 69 (5), 56 (16), 43 (17) and 36 (100).

Synthesis of 1,2,4-triazole-3-carboxylic acid 15[165, 166]



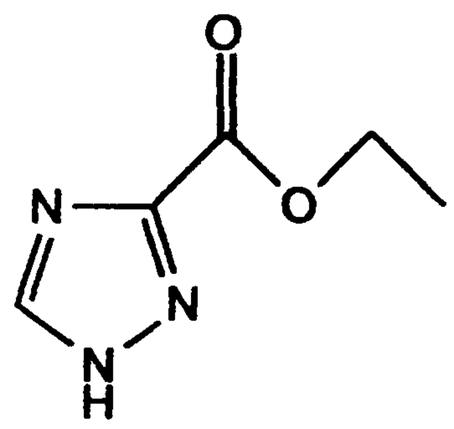
5-Amino-1,2,4-triazole-3-carboxylic acid **10** (8.5 g, 66 mmol) was heated to 50°C in water (52 ml) and concentrated hydrochloric acid (20 ml), cooled to 0°C with an ice/water bath, ice (60 g) was added and a precipitate formed. A solution of sodium nitrate (9.10 g, 130 mmol) in water (20 ml) was added dropwise maintaining the temperature below 5°C and left stirring for 1 h. The white solid was filtered off, pressed dry and immediately suspended in methanol (60 ml) and heated carefully with stirring to 40°C when a very exothermic reaction occurred and the solution was rapidly cooled in an acetone/dry ice bath. The solid was filtered off and dried to give 1,2,4-triazole-3-carboxylic acid **15** (7.07 g, 94%) of a colourless powder, m.p. 136-137°C (lit.,[166] 137°C).

Synthesis of methyl 1,2,4-triazole-3-carboxylate 16[166]



1,2,4-Triazole-3-carboxylic acid **15** (1.60 g, 14 mmol) was dissolved in methanol (30 ml) and the solution was saturated with hydrogen chloride gas at 0°C. The reaction was stirred at room temperature for 3 d and followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 m, elution with 2% acetonitrile/10 mM ammonium acetate). Nitrogen was bubbled through the solution to remove excess hydrogen chloride gas. The solvent was removed *in vacuo* and the hydrogen chloride salt of the product was recrystallised from water to give methyl 1,2,4-triazole-3-carboxylate **16** (1.60 g, 89%) as colourless crystals, m.p. 198°C (lit.,[164] 198°C); ν_{max} (nujol mull)/cm^{-1} 1724 (C=O); δ_H (400 MHz; DMSO-d^6) 4.13 (3 H, s, Me) and 8.67 (1 H, s, H-5); δ_C (400 MHz; DMSO-d^6) 52.16 (Me), 146.10 (C-5), 159.97 (C-3) and 163.07 (C=O); *m/z* (CI) 145 ($(M+NH_4)^+$, 12%), 128 ($(M+H)^+$, 100), 116 (14), 70 (34), 58 (11) and 41 (3); *m/z* (EI) 127 ($M^{+\cdot}$, 21%), 97 (58), 69 (100), 58 (26) and 42 (38).

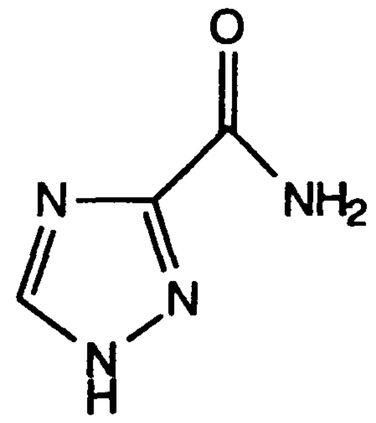
Synthesis of ethyl 1,2,4-triazole-3-carboxylate 17[167]



1,2,4-Triazole-3-carboxylic acid **15** (7.01 g, 62.5 mmol) was suspended in ethanol (100 ml) cooled to 0°C by an ice/water bath and saturated with hydrogen chloride gas for 30 min, then sealed and left stirring at room temperature for 7 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo*, the residue was treated with saturated aqueous sodium hydrogen

carbonate (41 ml) and the white precipitate collected, washed with water and recrystallised from ethanol to give 1.98 g of colourless crystals. The filtrate was extracted with ethyl acetate (3 x 50 ml), the organic extracts combined and dried over sodium sulfate, filtered and evaporated to give on recrystallisation 2.85 g. The total yield of ethyl 1,2,4-triazole-3-carboxylate **17** was 4.83 g (50%), m.p. 177°C (lit.,[167] 178°C); λ_{max} (H_2O)/nm 192.3; δ_H (250 MHz; DMSO-d^6) 1.29 (3 H, t, *J* 7.11, Me), 4.32 (2 H, q, *J* 7.07, CH_2), 8.71 (1 H, br s, H-5) and 14.96 (1 H, br s, H-1); δ_C (250 MHz; DMSO-d^6) 14.28 (Me), 61.20 (CH_2CH_3), 145.31 (C-5), 154.29 (C-3) and 159.96 (C=O); *m/z* (CI) 142 ($(M+H)^+$, 100%), 128 (10), 114 (9), 96 (11) and 70 (10); *m/z* (EI) 141 ($M^{+\cdot}$, 10%), 114 (33), 96 (90), 69 (100), 55 (10), 45 (10) and 42 (54).

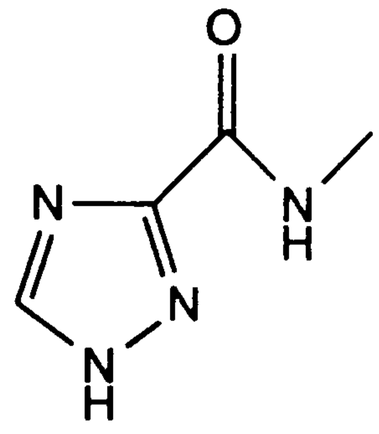
Synthesis of 1,2,4-triazole-3-carboxamide 18[166]



Ethyl 1,2,4-triazole-3-carboxylate **17** (0.210 g, 1.49 mmol) was dissolved in methanol (10 ml) and saturated aqueous ammonia (20 ml, d = 0.88) was added and the solution stirred at room temperature for 4 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was evaporated *in vacuo* and the residue gave on recrystallisation from methanol and water 1,2,4-triazole-3-carboxamide **18** (0.157 g, 94%) as colourless crystals, m.p. 312°C (lit.,[166] 312°C); ν_{max} (nujol mull)/cm^{-1} 3327 (NH-amide), 3123 (NH-amide) and 1699 (C=O); δ_H (250 MHz; DMSO-d^6) 7.73 (1 H, br s, NH-amide), 8.00 (1 H, br s, NH-amide),

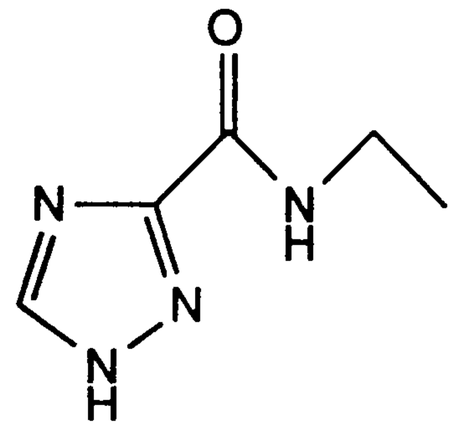
8.43 (1 H, s, H-5) and 14.59 (1 H, br s, H-1); *m/z* (CI) 130 ($(M+NH_4)^+$, 33%), 113 ($(M+H)^+$, 100), 96 (2), 84 (2) and 70 ({$(M+H)^+$ - $CONH_2$}, 3); *m/z* (EI) 112 ($M^{+\cdot}$, 33%), 96 (1), 84 (13), 69 ($(M^{+\cdot}$ - $CONH_2$), 100), 57 (18) and 42 (34).

Synthesis of *N*-methyl-1,2,4-triazole-3-carboxamide 19*



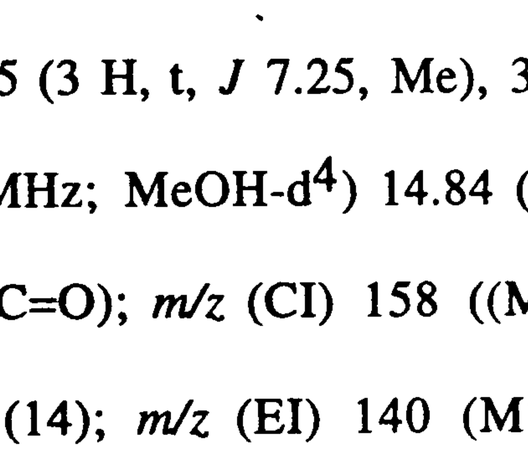
Methyl 1,2,4-triazole-3-carboxylate **16** (0.099 g, 0.78 mmol) was dissolved in methanol (10 ml) cooled to 0°C and saturated with methylamine gas. The solution was stirred at room temperature for 11 d and the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 10 mM ammonium acetate). The solvent was evaporated *in vacuo* and the residue gave on recrystallisation from methanol *N*-methyl-1,2,4-triazole-3-carboxamide **19** (0.79 g, 81%) as colourless crystals, m.p. 253-255°C; (Found: C, 37.86; H, 4.77; N, 44.13. $C_4H_6N_4O$ requires C, 38.13; H, 4.80; N, 44.41); δ_H (250 MHz; DMSO-d^6) 2.75 (1.5 H, s, Me), 2.77 (1.5 H, s, Me), 8.40 (1 H, s, H-5), 8.55 (0.5 H, br s, NH-amide) and 8.57 (0.5 H, br s, NH-amide); δ_C (250 MHz; DMSO-d^6) 25.72 (Me), 147.25 (C-5), 153.70 (C-3) and 158.65 (C=O); *m/z* (CI) 144 ($(M+NH_4)^+$, 5%), 127 ($(M+H)^+$, 23), 35 (8) and 18 (100); *m/z* (EI) 126 ($M^{+\cdot}$, 75%), 98 (32), 96 (53), 83 (25), 69 (97), 58 (81), 42 (100), 28 (87) and 17 (10).

Synthesis of *N*-ethyl-1,2,4-triazole-3-carboxamide 20*



Methyl 1,2,4-triazole-3-carboxylate **16** (0.40 g, 3.147 mmol) and ethylamine (2 ml, 70% (w/v) solution in water) were dissolved in methanol (2 ml) and stirred at 50°C for 3 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol and dichloromethane to give *N*-ethyl-1,2,4-triazole-3-carboxamide **20** (0.41 g, 92%) as colourless crystals, m.p. 188-190°C ; (Found: M^+ 140.0689. $C_5H_8N_4O$ requires 140.0699); δ_H (250 MHz; MeOH-d^4) 1.25 (3 H, t, *J* 7.25, Me), 3.46 (2 H, q, *J* 7.25, CH_2) and 8.49 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 14.84 (Me), 35.20 (CH_2), 146.99 (C-5), 156.43 (C-3) and 160.84 (C=O); *m/z* (CI) 158 ($(M+NH_4)^+$, 7%), 141 ($(M+H)^+$, 100), 113 (5), 96 (3) and 44 (14); *m/z* (EI) 140 ($M^{+\cdot}$, 3%), 125 (12), 96 (45), 83 (12), 69 (63), 44 (100) and 42 (84).

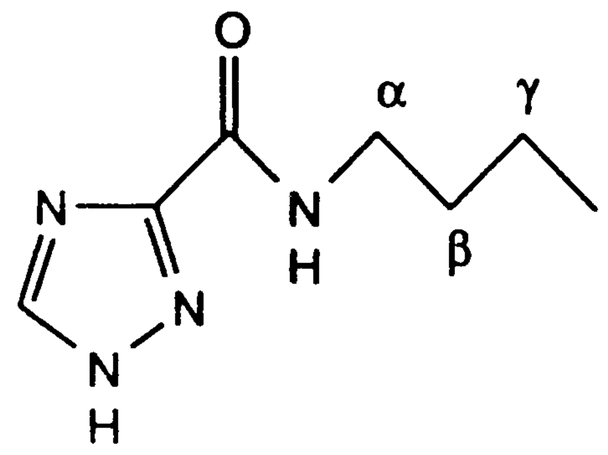
Synthesis of *N*-n-propyl-1,2,4-triazole-3-carboxamide 21*



Methyl 1,2,4-triazole-3-carboxylate **16** (0.40 g, 3.147 mmol) and n-

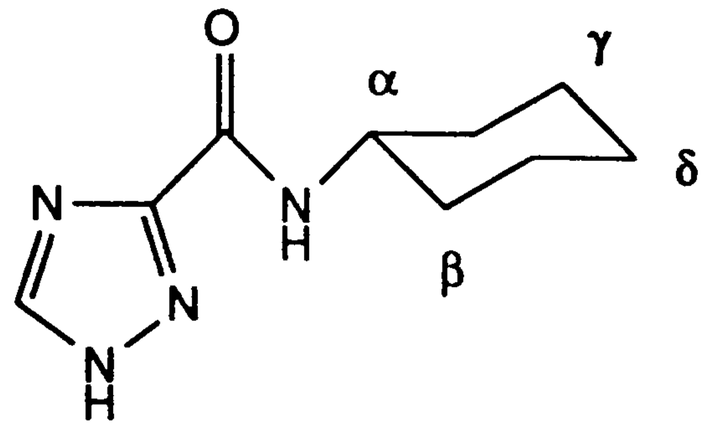
propylamine (2 ml) were dissolved in methanol (2 ml) and stirred at 50°C for 6 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol to give *N*-n-propyl-1,2,4-triazole-3-carboxamide **21** (0.35 g, 72%) as colourless crystals, m.p. 189-190°C; (Found: M^+ 154.0851. $C_6H_{10}N_4O$ requires 154.0856); δ_H (250 MHz; MeOH-d^4) 0.86 (3 H, t, *J* 7.43, Me), 1.53 (2 H, sextet, *J* 7.31, CH_2CH_3), 3.25 (2 H, t, *J* 7.03, $CH_2CH_2CH_3$) and 8.33 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 11.65 (Me), 23.67 (CH_2CH_3), 42.07 ($CH_2CH_2CH_3$), 147.06 (C-5), 156.35 (C-3) and 160.97 (C=O); *m/z* (CI) 172 ($(M+NH_4)^+$, 3%), 155 ($(M+H)^+$, 100), 141 (2), 125 (8), 113 (7), 96 (4), 70 (3) and 58 (7); *m/z* (EI) 154 ($M^{+\cdot}$, 12%), 125 (97), 96 (100), 91 (10), 83 (12), 81 (14), 69 (37), 58 (41), 55 (23) and 41 (33).

Synthesis of *N*-n-butyl-1,2,4-triazole-3-carboxamide 22*



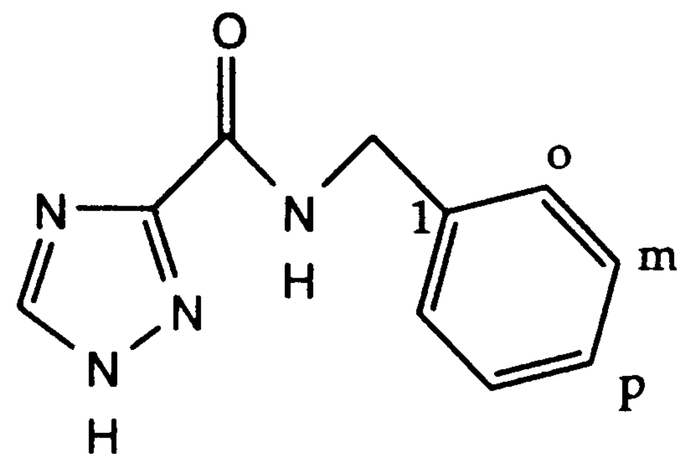
Ethyl 1,2,4-triazole-3-carboxylate **17** (0.210 g, 1.49 mmol) was dissolved in methanol (10 ml) and freshly distilled n-butylamine (20 ml) was added and the solution stirred at room temperature for 4 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solution was evaporated *in vacuo* to give on recrystallisation from methanol and dichloromethane *N*-n-butyl-1,2,4-triazole-3-carboxamide **22** (0.198 g, 79%) as colourless crystals, m.p. 185-187°C; (Found: C, 50.01; H, 7.09; N, 33.12. $C_7H_{12}N_4O$ requires C, 49.99; H, 7.19; N, 33.30); δ_H (250 MHz; MeOH-d^4) 1.00 (3 H, t, *J* 7.26, Me), 1.44 (2 H, sextet, *J* 7.32, CH_2-γ), 1.64 (2 H, quintet, *J* 7.33, CH_2-β), 3.43 (2 H, t, *J* 7.11, CH_2-α) and 8.46 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 14.09 (Me), 21.09 (*C*H_2-γ), 32.57 (*C*H_2-β), 40.09 (*C*H_2-α), 147.08 (C-5), 156.33 (C-3) and 160.89 (C=O); *m/z* (EI FAB) 169 ($(M+H)^+$, 75%), 110 (11) and 58 (48).

Synthesis of *N*-cyclohexyl-1,2,4-triazole-3-carboxamide 23*



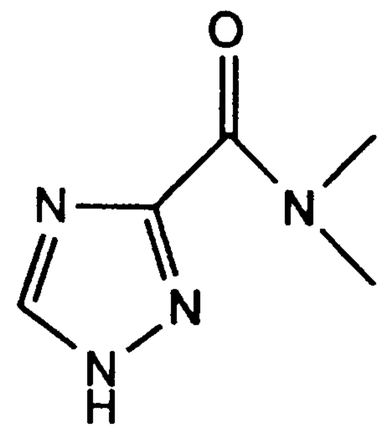
Methyl 1,2,4-triazole-3-carboxylate **16** (50 mg, 0.39 mmol) and cyclohexylamine (0.047 ml, 0.39 mmol) were dissolved in methanol (5 ml) and refluxed for 2 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol to give ***N*-cyclohexyl-1,2,4-triazole-3-carboxamide 23** (28 mg, 37%) as light yellow crystals, m.p. 162-164°C; (Found: C, 55.38; H, 7.48; N, 29.06. $C_9H_{14}N_4O$ requires C, 55.66; H, 7.27; N, 28.84); δ_H (250 MHz; MeOH-d^4) 1.39 (4 H, m, C*H*$_2$-γ, C*H*$_2$-δ), 1.72 (2 H, m as d, C*H*$_2$-γ), 1.86 (2 H, m, C*H*$_2$-β), 2.05 (2 H, m, C*H*$_2$-β), 3.13 (1 H, nonet, C*H*-α) and 8.17 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 25.42 (*C*H$_2$-δ), 25.95 (*C*H$_2$-γ), 31.97 (*C*H$_2$-β), 51.43 (*C*H-α), 149.12 (C-5), 157.03 (C-3) and 164.38 (C=O); *m/z* (CI) 195 ($(M+H)^+$, 38%), 166 (2), 151 (3), 137 (4), 98 (8) and 18 (100); *m/z* (EI) 194 ($M^{+\cdot}$, 23%), 166 (30), 151 (67), 137 (82), 123 (23), 98 (100), 69 (58) and 28 (15).

Synthesis of *N*-benzyl-1,2,4-triazole-3-carboxamide 24*



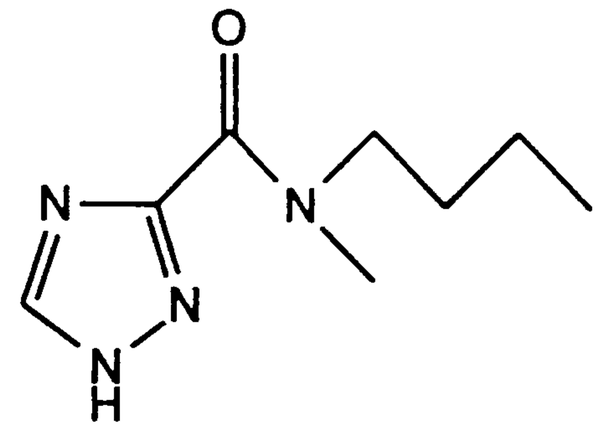
Methyl 1,2,4-triazole-3-carboxylate **16** (50 mg, 0.39 mmol), benzylamine (0.043 ml, 0.39 mmol) were dissolved in methanol (8 ml) and refluxed for 3 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue suspended in methanol. The colourless solid was filtered off and washed with methanol to give *N*-benzyl-1,2,4-triazole-3-carboxamide **24** (20 mg, 25%) as a colourless powder, m.p. 238-240°C; (Found: $M^{+\cdot}$ 202.0850. $C_{10}H_{10}N_4O$ requires 202.0856); δ_H (400 MHz; TFA) 5.22 (2 H, s, CH_2), 7.80 (6 H, m, H-aromatic, H-5) and 10.14 (1 H, br s, H-1); δ_C (400 MHz; TFA) 44.83 (CH_2), 127.75 (C-aromatic-o), 128.59 (C-aromatic-m), 128.95 (C-aromatic-p), 134.49 (C-aromatic-1), 143.28 (C-5), 148.45 (C-3) and 155.01 (C=O); *m/z* (CI) 203 ($(M+H)^+$, 100%), 159 (13), 106 (100), 96 (9), 91 (46), 77 (13), 65 (21) and 39 (13); *m/z* (EI) 202 ($M^{+\cdot}$, 11%), 159 (17), 106 (100), 96 (5), 91 (19), 77 (13), 69 (17) and 39 (7).

Synthesis of *N,N*-dimethyl-1,2,4-triazole-3-carboxamide 25*



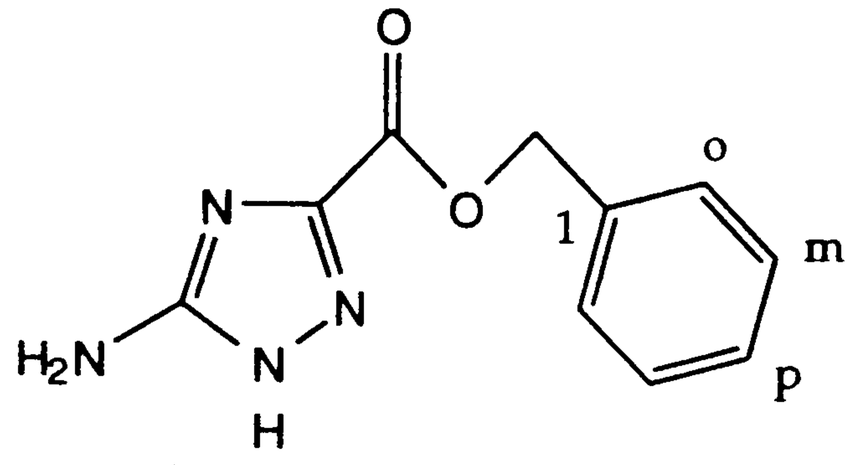
Methyl 1,2,4-triazole-3-carboxylate **16** (0.40 g, 3.15 mmol), dimethyl-amine (10 ml) and methanol (4 ml) were stirred at 50°C for 6 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol and dichloromethane to give *N,N*-dimethyl-1,2,4-triazole-3-carboxamide **25** (0.30 g, 68%) as colourless crystals, m.p. 163-165°C; (Found: $M^{+\cdot}$ 140.0697. $C_5H_8N_4O$ requires 140.0697); (Found: C, 42.67; H, 5.72; N, 40.22. $C_5H_8N_4O$ requires C, 42.86; H, 5.76; N, 39.97); δ_H (250 MHz; MeOH-d^4) 3.13 (3 H, s, Me, *syn*), 3.30 (3 H, s, Me, *anti*) and 8.48 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 36.11 (Me, *syn*), 39.09 (Me, *anti*), 146.58 (C-5), 156.02 (C-3) and 162.92 (C=O); *m/z* (CI) 158 ($(M+NH_4)^+$, 7%), 141 ($(M+H)^+$, 64), 124 (35), 96 (10), 79 (100), 61 (27) and 44 (15); *m/z* (EI) 140 ($M^{+\cdot}$, 10%), 111 (5), 96 (10), 83 (17), 72 (15), 69 (20) and 41 (100).

Synthesis of *N*-methyl-*N*-n-butyl-1,2,4-triazole-3-carboxamide 26*



Ethyl 1,2,4-triazole-3-carboxylate **17** (99 mg, 0.70 mmol) and freshly distilled *N*-methyl-n-butylamine (5 ml) was kept at 60°C for 10 d. The reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 5% acetonitrile/10 mM ammonium acetate) the solvent was removed *in vacuo* and the residue was recrystallised from methanol and dichloromethane to give *N*-methyl-*N*-n-butyl-1,2,4-triazole-3-carboxamide **26** (51 mg, 40%) of a white powder, (Found: C, 52.52; H, 7.65; N, 31.02. $C_8H_{14}N_4O$ requires C, 52.74; H, 7.75; N, 30.74); δ_H (250 MHz; MeOH-d^4) 1.02 (3 H, t, *J* 7.25, Me-δ), 1.46 (2 H, sextet, *J* 6.98, CH_2-γ), 1.65 (2 H, quintet, *J* 7.02, CH_2-β), 3.29 (3 H, s, Me-N), 3.43 (2 H, t, *J* 6.98, CH_2-α) and 8.47 (1 H, s, C-5); δ_C (250 MHz; MeOH-d^4) 14.15 (Me-δ), 21.13 (CH_2-γ), 32.62, (CH_2-β), 40.13 (CH_2-α), 51.71 (Me-N), 147.11 (C-5), 150.43 (C-3) and 160.98 (C=O); *m/z* (CI) 219 ($(M+H)^+$, 100%), 173 (15), 133 (9), 112 (21), 91 (83), 65 (9) and 41 (12).

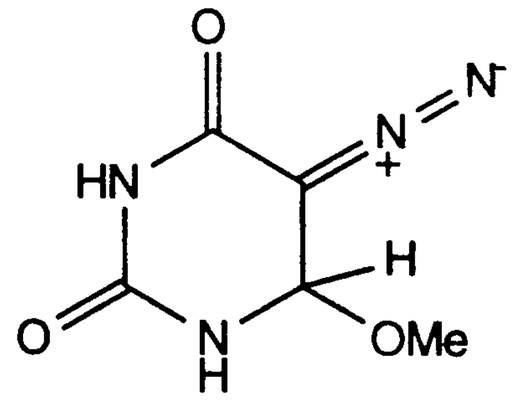
Synthesis of benzyl 5-amino-1,2,4-triazole-3-carboxylate 27[181]*



5-Amino-1,2,4-triazole-3-carboxylic acid **10** (0.64 g, 5 mmol) was suspended in phosphorous oxychloride (25 ml). Phosphorous pentachloride (1.25 g, 6 mmol) was added, the solution was stirred at room temperature for 2.5 h and then at 55°C for 45 min. The phosphorous oxychloride was removed under vacuum distillation. Benzyl alcohol (20 ml) and dimethylaminopyridine (10 mg) were added to the dry yellow solid and the suspension was left stirring at room temperature for 12 h. The remaining benzyl alcohol was removed under vacuum at room temperature and a white solid gradually formed, which was filtered off, dried and recrystallised from methanol to give benzyl 5-amino-1,2,4-triazole-3-carboxylate **27** (163 mg, 15%) as a colourless crystalline solid, (Found: $M^{+\cdot}$ 218.0816. $C_{10}H_{10}N_4O_2$ requires 218.0805); δ_H (250 MHz; DMSO-d^6) 4.99 (2 H, br s, N*H*$_2$/H$_2$0), 5.32 (2 H, s, C*H*$_2$) and 7.31-7.41 (5 H, m, H-aromatic); δ_C (250 MHz; DMSO-d^6) 66.30 (*C*H$_2$), 128.36-128.60 (C-aromatic-o/m/p), 135.84 (C-aromatic-1), 150.52 (C-5), 153.30 (C-3) and 159.43 (C=O); *m/z* (CI) 219 ($(M+H)^+$, 100%), 173 (15), 133 (9), 112 (12), 91 (83), 65 (9) and 41 (12).

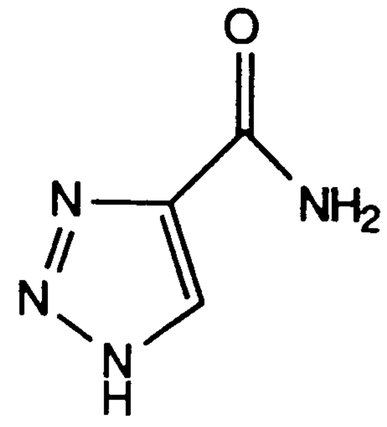
Synthesis of 1,2,3-Triazole Bases

Synthesis of 5-diazo-6-methoxy-1,6-dihydropyrimidin-2,4(1H,3H,6H)dione (5-diazouracil-6-methanolate) 29[182]



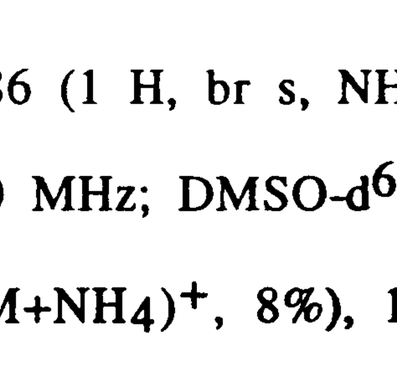
5-Aminouracil (0.173 g, 1.35 mmol) was dissolved in hydrochloric acid (3.4 ml, 1 M) cooled to 0°C with an ice/salt bath. Sodium nitrite (1.36 ml, 1 M) was added dropwise maintaining the temperature between 0°-3°C. The solution was stirred at room temperature for 1.5 h, then cooled to -10°C by an ice/salt bath and ethanol (2 x 9.50 ml, 95%) at -10°C was added. Rapid filtration of the precipitate and recrystallisation from boiling methanol (12 ml) gave 5-diazouracil-6-methanolate **29** (0.182 g, 79%) as yellow crystals, m.p. 198°C (lit.,[182] 198°C); λ_{max} (MeOH)/nm 210.9 and 263, (lit.,[182] 262 nm); δ_H (250 MHz; DMSO-d^6) 3.19 (3 H, s, OMe), 5.75 (1 H, d, *J* 4.07, H-6), 8.70 (1 H, br d, *J* 2.33, H-1) and 10.35 (1 H, s, H-3); δ_C (250 MHz; DMSO-d^6) 51.83 (OMe), 57.55 (C-6), 77.38 (C-5), 152.01 (C-2) and 162.88 (C-4); *m/z* (EI) 137 (($M^{+\cdot}$ - OMe), 43%), 112 (($M^{+\cdot}$ - N_2 - OMe), 52), 66 (42) and 31 ($OMe^{+\cdot}$, 100).

Synthesis of 1,2,3-triazole-4-carboxamide 30[184]



5-Diazouracil-6-methanolate **29** (1 g, 5.9 mmol) was dissolved in a mixture of acetonitrile (250 ml) and water (10 ml) at 50°C. The solution was sealed in a stainless steel reaction vessel heated at 100°C (internal temperature and pressure) for 18 h. When cooled, the solution and washings were filtered and the filtrate evaporated *in vacuo*. The yellow solid was recrystallised from glacial acetic acid (15 ml) to give 1,2,3-triazole-4-carboxamide **30** (0.28 g, 84.7%) as yellow crystals, m.p. 256-258°C (lit.,[184] 256-259°C); λ_{max} (H_2O)/nm 199 (lit.,[184] 197nm); δ_H (400 MHz; DMSO-d^6) 7.49 (1 H, br s, NH-amide), 7.86 (1 H, br s, NH-amide), 8.30 (1 H, s, H-5) and 15.44 (1 H, br s, H-1); δ_C (400 MHz; DMSO-d^6) 129.48 (C-5), 141.81 (C-4) and 161.65 (C=O); *m/z* (CI) 130 ($(M+NH_4)^+$, 8%), 113 ($(M+H)^+$, 20), 35 (6) and 18 (100); *m/z* (EI) 112 ($M^{+\cdot}$, 87%), 96 (85), 69 (22) and 17 (100).

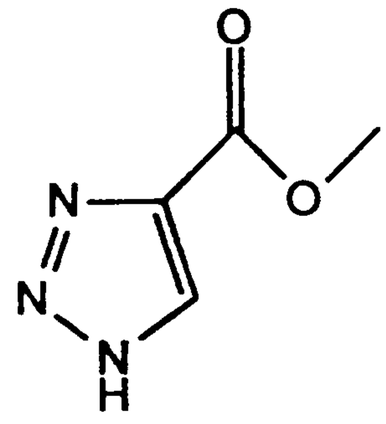
Synthesis (1) of methyl 1,2,3-triazole-4-carboxylate 35[188]



'Amberlyst' 15 strong acid cation exchange resin (1.4 g) was rinsed with methanol until the washings were neutral and dried *in vacuo*. 1,2,3-

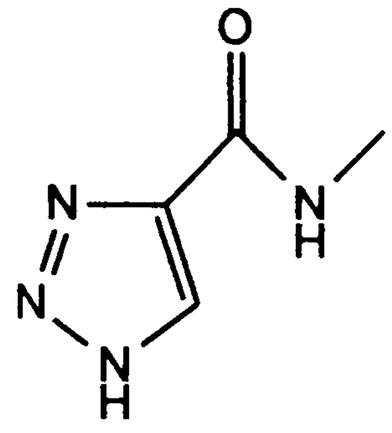
triazole-4-carboxamide **30** (100 mg, 0.892 mmol), methanol (8 ml) and the resin were gently stirred at reflux for 24 h. The progress of the reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solution was cooled, the resin collected in a column and eluted with methanol. The solvent was removed *in vacuo* to give after recrystallisation from water methyl 1,2,3-triazole-4-carboxylate **35** (64 mg, 56%) as colourless crystals, m.p. 145°C (lit.,[184] 144-145°C); δ_H (250 MHz; MeOH-d^4) 3.96 (3 H, s, Me) and 8.40 (1 H, s, H-5); *m/z* (CI) 145 ($(M+NH_4)^+$, 28%), 128 ($(M+H)^+$, 100), 110 (2) and 96 (10); *m/z* (EI) 127 ($M^{+\cdot}$, 14%), 96 (94), 69 (44), 59 (15) and 40 (100).

Synthesis (2) of methyl 1,2,3-triazole-4-carboxylate 35[189]



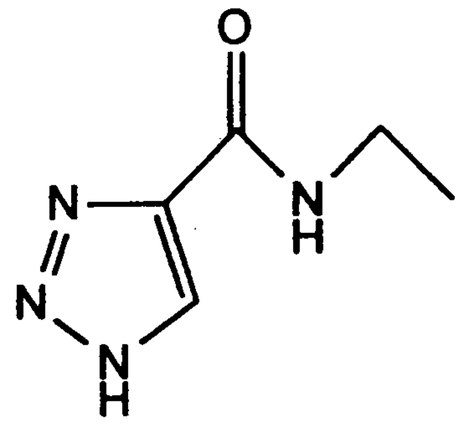
Trimethylsilyl azide (4.5 ml, 34 mmol) and methyl propiolate (2.0 ml, 23 mmol) were heated in a stainless steel autoclave at 130°C (internal pressure) for 19 h. After cooling, the trimethylsilyl group was hydrolysed by methanol (10 ml) and white crystals gradually formed. Evaporation of the mixture to dryness and recrystallisation of the residue from methanol with charcoal gave methyl 1,2,3-triazole-4-carboxylate **35** (2.38 g, 83%) as colourless crystals. NMR and analytical data was identical with methyl 1,2,3-triazole-4-carboxylate **35** synthesised from 1,2,3-triazole-4-carboxamide **30**.

Synthesis of *N*-methyl-1,2,3-triazole-4-carboxamide 38



Methyl 1,2,3-triazole-4-carboxylate **35** (107 mg, **0.84** mmol) in methylamine (5 ml, 30-40% (w/v) solution in water) was left at room temperature for 7 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol and dichloromethane to give *N*-methyl-1,2,3-triazole-4-carboxamide **38** (0.77 g, 76%) as colourless crystals, m.p. 260°C (lit.,[184] 258-261°C); δ_H (250 MHz; DMSO-d^6) 2.75 (1.5 H, s, Me), 2.77 (1.5 H, s, Me), 8.32 (1 H, s, H-5) and 8.47 (1 H, br s, NH-amide); δ_C (250 MHz; DMSO-d^6) 25.64 (Me), 128.83 (C-5), 142.09 (C-4) and 160.34 (C=O); *m/z* (CI) 144 ($(M+NH_4)^+$, 26%), 127 ($(M+H)^+$, 100), 96 (5), 84 (18) and 55 (5); *m/z* (EI) 126 ($M^{+\cdot}$, 90%), 100 (10), 96 (100), 82 (90), 69 (50), 54 (43) and 40 (38).

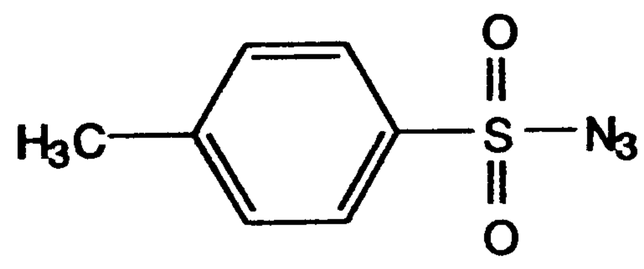
Synthesis of *N*-ethyl-1,2,3-triazole-4-carboxamide 39*



Methyl 1,2,3-triazole-4-carboxylate **35** (0.51 g, 4.01 mmol) and ethylamine

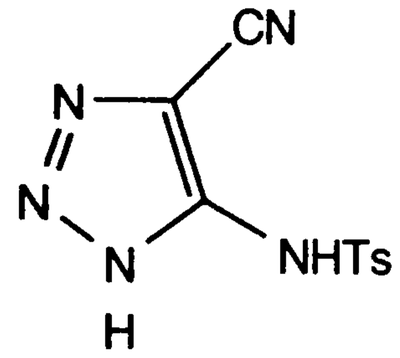
(3 ml, 70% (w/v) solution in water) were dissolved in methanol (2 ml) and stirred at 50°C for 2 d. The reaction was monitored by reverse phase HPLC (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). The solvent was removed *in vacuo* and the residue recrystallised from methanol and dichloromethane to give *N*-ethyl-1,2,3-triazole-4-carboxamide **39** (0.50 g, 89.5%) as colourless crystals, m.p. 148-150°C; (Found: $M^{+\cdot}$ 140.0698. $C_5H_8N_4O$ requires 140.0699); (Found: C, 42.56; H, 5.69; N, 39.73. $C_5H_8N_4O$ requires C, 42.86; H, 5.76; N, 39.97); δ_H (250 MHz; MeOH-d^4) 1.26 (3 H, t, *J* 7.25, Me), 3.43 (2 H, q, *J* 7.23, CH_2CH_3) and 8.23 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 14.95 (Me), 35.11 (CH_2CH_3), 130.30 (C-5), 142.93 (C-4) and 162.95 (C=O); *m/z* (CI) 158 ($(M+NH_4)^+$, 11%), 141 ($(M+H)^+$, 10), 128 (8), 113 (3), 85 (3), 79 (5), 58 (6) and 44 (7); *m/z* (EI) 140 ($M^{+\cdot}$, 38%), 125 (23), 96 (90), 83 (6), 77 (51), 69 (29), 57 (8), 44 (100) and 40 (18).

Synthesis of p-toluenesulfonylazide 40[237]



Sodium azide (4.13 g, 63.5 mmol) and p-toluenesulfonylchloride (10 g, 52.5 mmol) and ethanol (60 ml, 95%) were stirred at room temperature for 1 h, then poured into water (200 ml). The separated oily sulfonylazide was washed with water and dried over sodium sulfate to give p-toluenesulfonylazide **40** (5.53 g, 54%) as a clear, colourless oil which was used without further purification.

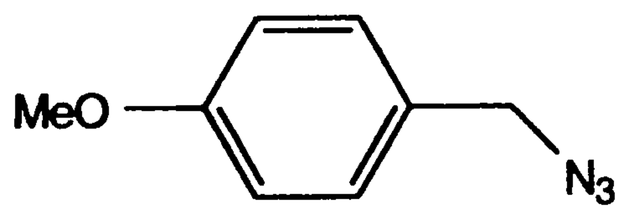
Synthesis of 1,2,3-triazole-4-cyano-5-*N*-toluenesulfonyl-amine 48[191, 192]



Ts = $CH_3C_6H_4SO_2$-

To a cooled solution (0°C) of malononitrile (0.66 g, 10 mmol) and sodium hydroxide (7.22 ml, 20 mM) was added dropwise p-toluenesulfonyl azide **40** (1.97 g, 10 mmol) in ether (8 ml). The mixture was stirred vigorously for 1 h. The reaction was washed with ether (3 x 20 ml) and the aqueous layer was brought to pH 0.75 with concentrated sulfuric acid. The precipitate formed was filtered off and recrystallised from water to give 1,2,3-triazole-4-cyano-5-*N*-toluenesulfonylamine **48** (2.15 g, 82%) as colourless crystals, m.p. 190°C (lit.,[191] 190°C); δ_H (250 MHz; MeOH-d^4) 2.44 (3 H, s, Me) and 7.56 (4 H, ABq, *J* 8.30, H-aromatic); δ_C (250 MHz; MeOH-d^4) 21.48 (Me), 111.82 (C-5), 115.60 (CN), 128.32 (C-aromatic-o), 130.75 (C-aromatic-m), 137.61 (C-aromatic-p), 145.79 (C-aromatic-1) and 146.14 (C-4); *m/z* (CI) 281 ($(M+NH_4)^+$, 100%), 264 ($(M+H)^+$, 37), 238 (43), 155 (58), 139 (44), 124 (10), 108 (32) and 91 (43); *m/z* (EI) 263 ($M^{+\cdot}$, 12%), 155 (50), 124 (3), 91 (100), 77 (3), 65 (28), 53 (9) and 39 (12).

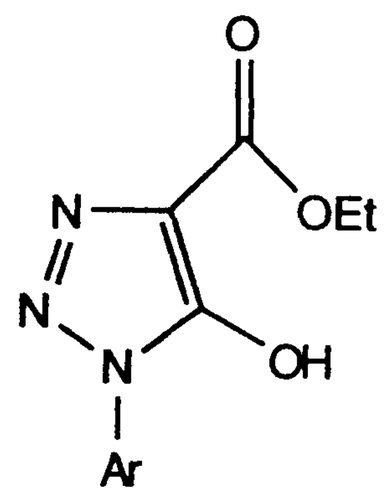
Synthesis of 4-methoxybenzyl azide 49[197]



Sodium azide (2.15 g, 33 mmol) was stirred for 24 hrs at 20°C with a solution

of 4-methoxybenzyl chloride (5.18 g, 33 mmol) in dry DMF (20 ml). The reaction was quenched with water (20 ml). The organic azide was extracted into ether and the extracts were washed well with water and dried with magnesium sulfate. Evaporation *in vacuo* (<20°C) afforded the title azide, 4-methoxybenzyl azide **49** (5.12 g, 95%) as a colourless, clear oil which was used without further purification, ν_{max} (film)/cm^{-1} 2000.

Synthesis of ethyl 5-hydroxy-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate 50[197]

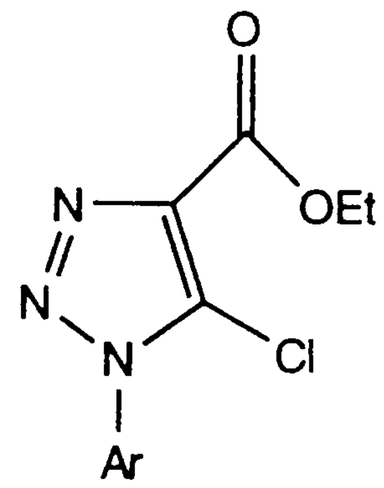


Ar = 4-$CH_3OC_6H_4CH_2$-

Sodium (6.03 g, 0.26 mol) was added to dry ethanol (450 ml) followed by diethyl malonate (41.9 g, 0.26 mol). After 30 min a solution of 4-methoxybenzyl azide **49** (42.5 g, 0.26 mol) in ethanol (50 ml) was added dropwise with stirring and the mixture was gently refluxed for 18 h. When cool, most of the solvent was removed *in vacuo* and water was added. Acidification to pH 2 with dilute hydrochloric acid gave a crystalline precipitate. The solid was filtered off, washed with water, dried in an evacuated desiccator over phosphorous pentoxide and recrystallised from chloroform and petroleum ether (40°-60°C) to give ethyl 5-hydroxy-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **50** (45.0 g, 65%) as colourless crystals, m.p. 116°C (lit.,[197] 117°C); δ_H (250 MHz; MeOH-d^4) 1.36 (3 H, t, *J* 7.11, Me), 3.77 (3 H, s, OMe), 4.31 (2 H, q, *J* 7.17, C*H*$_2$CH$_3$), 5.12 (2 H, s, C*H*$_2$N)

and 7.06 (4 H, ABq, *J* 8.73, H-aromatics); δ_C (250 MHz; MeOH-d^4) 14.89 (Me), 47.36 (CH_2N), 55.62 (OMe), 60.42 (CH_2CH_3), 114.77 (C-aromatic-m), 118.81 (C-4), 129.90 (C-aromatic-o), 130.66 (C-aromatic-1), 160.47 (*C*OMe), 161.18 (C-5) and 165.28 (C=O); *m/z* (CI) 295 ($(M+NH_4)^+$, 20%), 278 ($(M+H)^+$, 100), 252 (81), 206 (5), 176 (5), 136 (18) and 121 (25); *m/z* (EI) 277 ($M^{+\cdot}$, 8%), 205 ($(M^{+\cdot}$ - COOEt), 33), 176 (43), 136 (33), 121 (100) and 78 (18).

Synthesis of ethyl 5-chloro-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate 51[197]

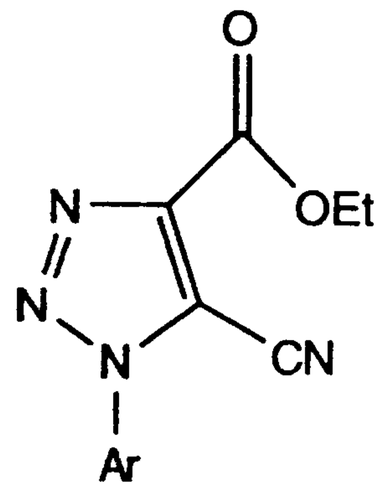


Ar = 4-$CH_3OC_6H_4CH_2$-

Phosphorous pentachloride (1.90 g, 9.12 mmol) was added to a stirred solution of the hydroxy ester **50** (2.34 g, 8.44 mmol) in dry toluene (25 ml) and the mixture was stirred at 40°C for 90 min. The solvent was removed *in vacuo* and the residue taken up in ether and washed well with saturated sodium hydrogen carbonate, water, dried over magnesium sulfate and evaporated to give on recrystallisation from ether and petroleum ether (40°-60°C) ethyl 5-chloro-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **51** (1.66 g, 68%) as colourless crystals, m.p. 76°C (lit.,[197] 77°C); δ_H (250 MHz; $CDCl_3$) 1.38 (3 H, t, *J* 7.13, Me), 3.78 (3 H, s, OMe), 4.41 (2 H, q, *J* 7.13, CH_2CH_3), 5.48 (2 H, s, CH_2N) and 7.05 (4 H, ABq, *J* 8.73, H-aromatic); δ_C (250 MHz; $CDCl_3$) 14.17 (Me), 51.84 (CH_2N), 55.26 (OMe), 61.43 (CH_2CH_3), 114.35 (C-aromatics-m), 125.04 (C-aromatics-o), 129.52 (C-aromatics-1),

135.07 (C-4/5), 159.63 (*C*OMe) and 159.95 (C=O); *m/z* (CI) 315 ($(M+2+NH_4)^+$, 3%), 313 ($(M+NH_4)^+$, 9), 298 ($(M+2+H)^+$, 10), 296 ($(M+H)^+$, 33), 278 (3), 138 (9) and 121 (100); *m/z* (EI) 297 ($(M+2)^{+\cdot}$, 2%), 295 ($M^{+\cdot}$, 5), 223 (10), 194 (5), 178 (37), 160 (17), 137 (13), 121 (100) and 78 (13).

Synthesis of ethyl 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate 52[197]

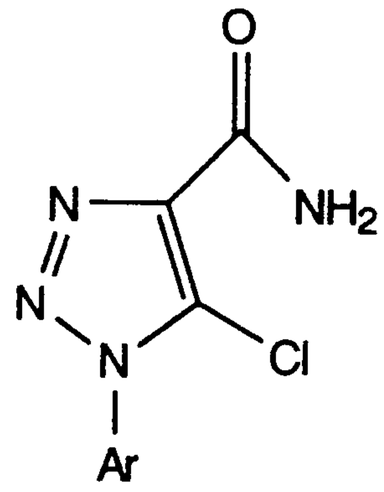


Ar = 4-$CH_3OC_6H_4CH_2$-

Sodium cyanide (0.187 g, 3.80 mmol) was added to a solution of the chloro ester **51** (1.06 g, 3.66 mmol) in dry DMF (10 ml) and stirred at 80°C for 24 h. When cool, the solvent was removed *in vacuo* and the oil partitioned between ethyl acetate (100 ml) and water (25 ml). The organic layer was washed with water (3 x 25 ml), brine (25 ml), dried over magnesium sulfate and evaporated to give on recrystallisation from ethanol ethyl 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **52** (0.54 g, 52%) as colourless crystals, m.p. 82°C (lit.,[197] 81-83°C); δ_H (250 MHz; $CDCl_3$) 1.41 (3 H, t, *J* 7.08, Me), 3.81 (3 H, s, OMe), 4.46 (2 H, q, *J* 7.06, CH_2CH_3), 5.65 (2 H, s, CH_2N) and 7.13 (4 H, ABq, *J* 8.55, H-aromatic); δ_C (250 MHz; $CDCl_3$) 13.83 (Me), 54.30 (CH_2N), 55.08 (OMe), 62.25 (CH_2CH_3), 107.17 (CN), 114.41 (C-aromatic-m), 124.07 (C-aromatic-o), 124.87 (C-5), 129.97 (C-aromatic-1), 141.01 (C-4), 157.92 (C=O) and 160.50 (*C*OMe); *m/z* (CI) 287 ($(M+H)^+$, 5%), 185 (5) and 121 (100); *m/z* (EI) 286 ($M^{+\cdot}$, 25%), 229 (11), 185 (22), 121 (100) and

78 (32).

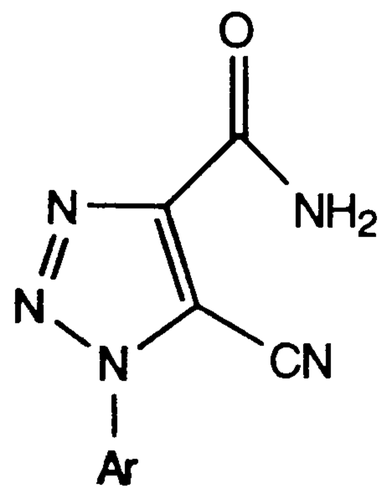
Synthesis of 5-chloro-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxamide 53*



Ar = 4-$CH_3OC_6H_4CH_2$-

The chloro ester **51** (0.100 g, 0.361 mmol) was dissolved in methanol previously saturated with ammonia at 0°C. The solution was left at room temperature for 6 d and monitored by TLC. The solvent was removed *in vacuo* to give after recrystallisation from dichloromethane and petroleum ether (40°-60°C) 5-chloro-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxamide **53** (0.076 g, 79%) as colourless crystals, m.p. 168-169°C; (Found: $(M+2)^{+\cdot}$ 268.0542. $C_{11}H_{11}N_4O_2{}^{37}Cl$ requires 268.0542); δ_H (250 MHz; $CDCl_3$) 3.72 (3 H, s, OMe), 5.41 (2 H, s, CH_2N), 6.63 (1 H, br s, NH-amide), 6.98 (1 H, br s, NH-amide) and 7.00 (4 H, ABq, *J* 14.5, H-aromatic); δ_C (250 MHz; $CDCl_3$) 51.71 (CH_2N), 55.29 (OMe), 114.40 (C-aromatic-m), 125.10 (C-aromatic-o), 129.58 (C-aromatic-1), 136.23 (C-4/5), 159.98 (*C*OMe) and 161.02 (C=O); *m/z* (CI) 286 ($(M+2+NH_4)^+$, 6%), 284 ($(M+NH_4)^+$, 25), 269 ($(M+2+H)^+$, 8), 267 ($(M+H)^+$, 17), 157 (25), 138 (20), 121 (100), 96 (60) and 79 (65); *m/z* (EI) 268 ($(M+2)^{+\cdot}$, 15%), 266 ($M^{+\cdot}$, 50), 237 (17), 203 (42), 159 (30), 133 (50), 121 (100) and 78 (35).

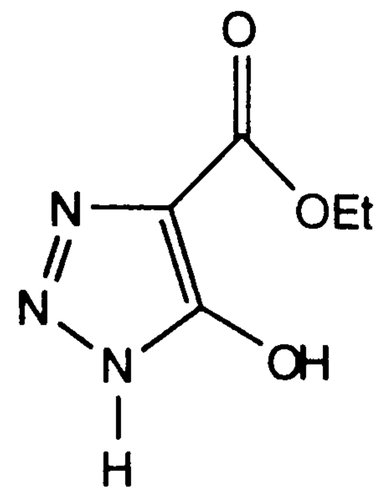
Synthesis of 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxamide 54*



Ar = 4-$CH_3OC_6H_4CH_2$-

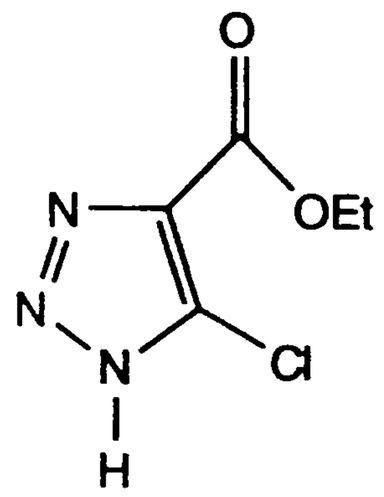
Ethyl 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **52** (190 mg, 0.66 mmol), concentrated aqueous ammonia (10 ml, **d** = 0.88) and methanol (5 ml) were left at room temperature in a sealed flask for 2 d. The reaction was monitored by TLC. The solvent was removed and the residue was recrystallised from acetone and petroleum ether (40°-60°C) to give 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxamide **54** (85 mg, 50%) as light yellow crystals, m.p. 113-115°C; (Found: $M^{+\cdot}$ 257.0904. $C_{12}H_{11}N_5O_2$ requires 257.0914); δ_H (250 MHz; $CDCl_3$) 3.89 (3 H, s, OMe), 5.77 (2 H,s, CH_2N), 6.33 (1 H, br s, NH-amide), 6.97 (4 H, ABq, *J* 8.70, H-aromatic) and 7.40 (1 H, br s, NH-amide); δ_C (250 MHz; $CDCl_3$) 54.87 (CH_2N), 55.22 (OMe), 114.12-158.29 (C-aromatic/C-4/5/CN), 159.57 (*C*OMe) and 162.74 (C=O); *m/z* (CI) 275 ($(M+NH_4)^+$, 15%), 258 ($(M+H)^+$, 10) and 121 (100); *m/z* (EI) 257 ($M^{+\cdot}$, 20%), 229 (8) and 121 (100).

Synthesis of ethyl 5-hydroxy-1,2,3-triazole-4-carboxylate 55[197]



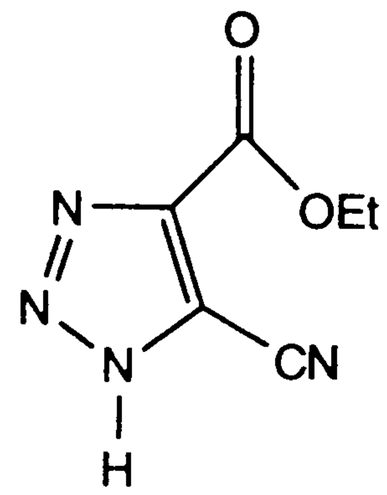
Ethyl 5-hydroxy-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **50** (200 mg, 0.72 mmol) in trifluoroacetic acid (2 ml) was stirred at 65°C (bath temperature) for 6 h. The course of the reaction was followed by TLC. The solution was cooled and the solvent removed *in vacuo*. Water (20 ml) was added to the brown residue, the solid was filtered and the filtrate was evaporated to dryness. Recrystallisation from ethyl acetate and petroleum ether (40°-60°C) to give ethyl 5-hydroxy-1,2,3-triazole-4-carboxylate **55** (52 mg, 46%) as colourless crystals, m.p. 143-145°C (lit.,[197] 145-146°C); δ_H (250 MHz; MeOH-d^4) 1.38 (3 H, t, *J* 7.10, Me) and 4.31 (2 H, q, *J* 7.10, CH_2CH_3); δ_C (250 MHz; MeOH-d^4) 14.92 (Me), 60.90 (CH_2CH_3), 118.69 (C-4), 162.82 (C-5) and 165.21 (C=O); *m/z* (CI) 175 ($(M+NH_4)^+$, 28%), 158 ($(M+H)^+$, 100), 149 (6), 132 (17), 86 (2) and 44 (2); *m/z* (EI) 157 ($M^{+\cdot}$, 38%), 136 (3), 112 (6), 69 (18), 58 (12) and 44 (100).

Synthesis of ethyl 5-chloro-1,2,3-triazole-4-carboxylate 59[197]



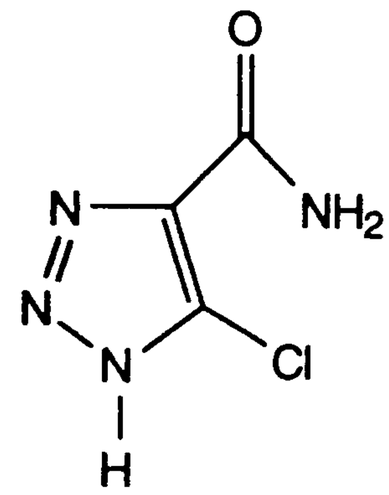
The chloro ester **51** (3.16 g, 10.7 mmol) in trifluoroacetic acid (25 ml) was heated at 67°C (oil bath temperature) for 3 h and the course of the reaction was followed by TLC. The solvent was removed *in vacuo* and water was added to the residue. The brown solid was filtered off and the filtrate evaporated to dryness and recrystallised from toluene and petroleum ether (40°-60°C) to give ethyl 5-chloro-1,2,3-triazole-4-carboxylate **59** (1.50 g, 90%) as colourless crystals, m.p. 77-79°C (lit.,[197] 78-81°C); δ_H (250 MHz; MeOH-d^4) 1.42 (3 H, t, *J* 7.12, Me) and 4.42 (2 H, q, *J* 7.08, CH_2CH_3); δ_C (250 MHz; MeOH-d^4) 14.43 (Me), 62.68 (CH_2CH_3), 133.94 (C-4), 139.15 (C-5) and 160.47 (C=O); *m/z* (CI) 195 ($(M+2+NH_4)^+$, 33%), 193 ($(M+NH_4)^+$, 100), 178 ($(M+2+H)^+$, 11), 176 ($(M+H)^+$, 40), 158 (22) and 148 (15); *m/z* (EI) 177 ($(M+2)^{+\cdot}$, 10%), 175 ($M^{+\cdot}$, 28), 147 (75), 130 ($(M^{+\cdot}$ - OEt), 100), 103 (32), 91 (11), 74 (34) and 47 (29).

Synthesis of ethyl 5-cyano-1,2,3-triazole-4-carboxylate 56[197]



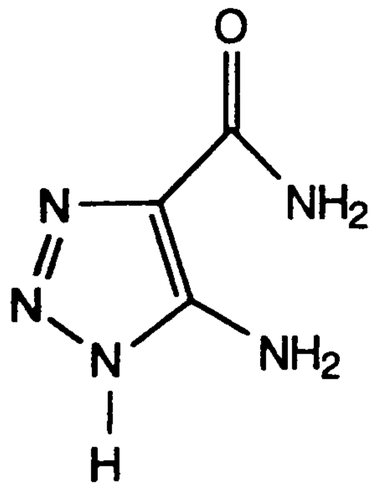
Ethyl 5-cyano-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxylate **52** (40 mg, 0.17 mmol) in trifluoroacetic acid (3 ml) was refluxed at 65°C (bath temperature) for 3.5 h. The reaction was followed by TLC. The solvent was removed *in vacuo* and the brown oil was suspended in water (20 ml). The brown solid was filtered and the filtrate was evaporated to dryness and recrystallised from ether and petroleum ether (40°-60°C) to give ethyl 5-cyano-1,2,3-triazole-4-carboxylate **56** (56 mg, 78%) as colourless crystals, m.p. 111-112°C (lit.,[197] 111°C-113°C); δ_H (250 MHz; $CDCl_3$) 1.46 (3 H, t, *J* 7.08, Me) and 4.56 (2 H, q, *J* 7.18, CH_2CH_3); δ_C (250 MHz; $CDCl_3$) 13.95 (Me), 63.55 (CH_2CH_3), 109.98 (CN), 122.96 (C-5), 141.54 (C-4) and 158.77 (C=O); *m/z* (CI) 184 ($(M+NH_4)^+$, 100%), 167 ($(M+H)^+$, 13) and 121 (90); *m/z* (EI) 166 ($M^{+\cdot}$, 12%), 139 (38), 121 (80) and 69 (38).

Synthesis of 5-chloro-1,2,3-triazole-4-carboxamide 58[197]



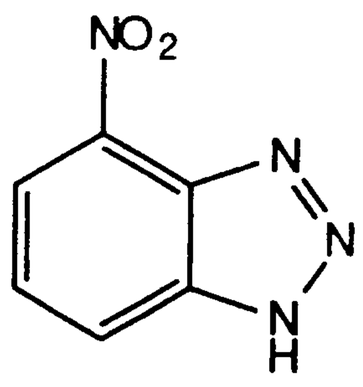
5-Chloro-1-(4-methoxybenzyl)-1,2,3-triazole-4-carboxamide **53** (67 mg, 0.23 mmol) in trifluoroacetic acid (3 ml) was refluxed at 65°C (bath temperature) for 3 h. The reaction was followed by TLC. The solvent was removed *in vacuo* and the solid residue was suspended in water (30 ml). The pale brown solid was filtered and the filtrate was evaporated to dryness to give on recrystallisation from methanol and dichloromethane 5-chloro-1,2,3-triazole-4-carboxamide **58** (28 mg, 82%) as colourless crystals, m.p. 158-161°C (decomp); λ_{max} (H_2O)/nm 192.1 and 232.0; δ_H (250 MHz; MeOH-d^4) 7.53 (1 H, br s, NH-amide) and 7.88 (1 H, br s, NH-amide); δ_C (250 MHz; MeOH-d^4) 137.26 (C-4), 137.69 (C-5) and 163.33 (C=O); *m/z* (CI) 166 ($(M+2+NH_4)^+$, 6%), 164 ($(M+NH_4)^+$, 18), 149 ($(M+2+H)^+$, 25) and 147 ($(M+H)^+$, 100); *m/z* (EI) 148 ($(M+2)^{+\cdot}$, 71%), 146 ($M^{+\cdot}$, 24), 132 (83), 130 (27) and 44 (100).

Synthesis of 5-amino-1,2,3-triazole-4-carboxamide 60



Ethyl 5-chloro-1,2,3-triazole-4-carboxylate **59** (128 mg, 1.24 mmol) was dissolved in methanol (50 ml), cooled to 0°C and saturated with ammonia gas. The solution was sealed and left for 10 d at room temperature. The solvent was evaporated and the residue was recrystallised from methanol to give 5-amino-1,2,3-triazole-4-carboxamide **60** (107 mg, 68%) as a colourless powder, m.p. 224°C (lit.,[198] 224-225°C); δ_H (400 MHz; MeOH-d^4) 7.33 (1 H, br s, NH-amide) and 7.64 (1 H, br s, NH-amide); δ_C (400 MHz; MeOH-d^4) 135.31 (C-4), 136.09 (C-5) and 165.34 (C=O); *m/z* (CI) 145 ($(M+NH_4)^+$, 13%), 128 ($(M+H)^+$, 100), 85 (5) and 58 (5); *m/z* (EI) 127 ($M^{+\cdot}$, 15%), 91 (10), 56 (63) 44 (65) and 28 (100).

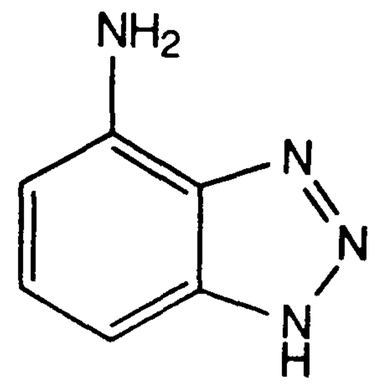
Synthesis of 4-nitro-benzotriazole 63[203]



Benzotriazole (10.0 g, 83 mmol) was dissolved in concentrated sulfuric acid (33 ml). The solution was cooled and concentrated nitric acid (6 ml) was added dropwise keeping the temperature below 30°C. The mixture was heated at 60°C (bath temperature) for 1 h. When cooled, water was added until no more precipitate developed. The solid was filtered off giving on recrystallisation from methanol and acetone 4-nitro-benzotriazole **63** (11.0 g, 84%) as light yellow crystals, m.p. 228°C (lit.,[203] 229°C); λ_{max} (H_2O)/nm 199.2 and 225.9; δ_H (400 MHz; DMSO-d^6) 7.63 (1 H, dd appears as t, *J* 8.00, H-6), 8.46 (1 H, d, *J* 7.80, H-5) and 8.59 (1 H, d, *J* 8.20, H-7); *m/z* (CI) 182 ($(M+NH_4)^+$, 3%), 165 ($(M+H)^+$, 12), 135 (100) and 52 (5); *m/z* (EI) 164 ($M^{+\cdot}$, 37%), 105 (25), 91 (38), 77 (38), 69 (53), 63 (96), 52 (60), 43 (100) and 38

(33).

Synthesis of 4-amino-benzotriazole 64[202]



A solution of 4-nitro-benzotriazole **63** (0.40 g, 2.98 mmol) in ethanol (40 ml) containing 10% palladium on carbon (80 mg) was hydrogenated at atmospheric pressure and temperature for 4 h. The catalyst was removed by filtration through a pad of celite and washed with ethanol. The solvent was removed *in vacuo* and the residue was recrystallised from ethanol and dichloromethane to give 4-amino-benzotriazole **64** (0.23 g, 70%) as yellow crystals, m.p. 149°C (lit.,[203] 149°C); λ_{max} (H_2O)/nm 191.1 and 218.9; δ_H (400 MHz; MeOH-d^4) 6.59 (1 H, d, *J* 7.50, H-5), 6.98 (1 H, d, *J* 8.10, H-7) and 7.25 (1 H, dd appears as t, *J* 7.81, H-6); *m/z* (EI) 134 ($M^{+\cdot}$, 100%), 105 (95), 79 (82), 69 (9), 57 (12), 52 (87) and 41 (12).

Enzymatic Synthesis of Nucleoside Analogues

Purification of Nucleoside *N*-Deoxyribosyltransferases (E.C. 2.4.2.6.) from *Lactobacillus leichmannii*

Lactobacillus leichmannii ATCC 4797 was grown in an MRS (DeMan-Rogosa-Sharpe) medium (52 gl^{-1}) previously sterilised by autoclaving at 120°C. The medium was inoculated with a 24 h starter culture (1%) grown in the same medium and incubated at 37°C for 24 h without aeration or agitation. The bacterial suspension was harvested by centrifugation (10

000 g) for 15 min at 4°C. The pellet produced was washed twice with 0.02 M PIPES buffer (pH 6.5) containing 0.02% (w/v) sodium azide and the cell paste stored at -20°C. Thawed cells were disrupted by three passes through a French pressure cell (20 000 psi) keeping the temperature below 10°C and the cell debris was removed by centrifugation (20 000 g) for 30 min at 4°C. Cell-free extracts were dialysed overnight against 100 volumes of 0.02 M PIPES buffer (pH 6.5) containing 0.02% (w/v) sodium azide. The final preparation could be stored at -20°C for up to 3 months without significant loss of activity.

Protein Determination

The concentration of protein was estimated using the dye-binding BioRad protein assay according to the method of Bradford.[238] The colorimetric property of Coomassie blue G-250 was employed. When bound to protein the absorbance maximum of the dye changes from 465 nm to 595 nm. Bovine serum albumin was used as the standard.

Definition of Unit and Specific Activity

One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1.0 μmol of product (2'-deoxyadenosine) formed in 1 min in the assay conditions from 2'-deoxycytidine and adenine. Specific activity was defined as units per milligram of protein.

Standard Assay of *N*-Deoxyribosyltransferase

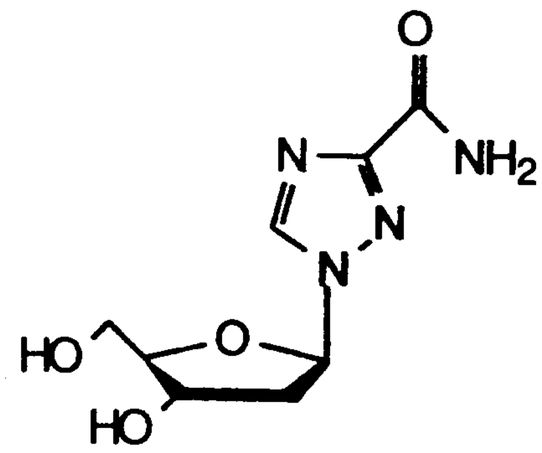
The standard reaction assay mixture contained 2'-deoxycytidine (1.5 mM) and adenine (0.5 mM) as donor and acceptor, respectively, in citrate

buffer (50 mM, pH 6.0). The reactions were initiated by the addition of a volume of the crude enzyme preparation equivalent to 50 μg/ml of protein. The final volume of the reaction mixture was 0.5 ml and it was incubated at 40°C with no shaking. At intervals of 0, 5, 10, 15, 20 and 30 min a 20 μl aliquot was removed from the reaction mixture and analysed by HPLC. The rate of deoxyribosyl transfer from the pyrimidine nucleoside to the purine base was measured by following the formation of 2'-deoxyadenosine. The concentration of nucleosides and bases present in the reaction mixture was determined using reverse phase HPLC on a Techsphere 5C8 column (25 cm x 4.6 mm and a precolumn, 5 cm x 4.6 mm), using a mobile phase of acetonitrile and doubly distilled water (5:95) at a flow rate of 1.2 ml/min and detected by UV at 254 nm.

Analytical Studies

The triazole base (0.05 ml, 10mM), 2'-deoxycytidine or thymidine (0.15 ml, 10mM) in citrate buffer (0.20 ml, 50 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (0.05 ml, 10% (v/v)) was allowed to equilibrate for 10 min at 40°C. The reaction was initiated by the addition of the crude *N*-deoxyribosyltransferase extract (0.05 ml, 15.8 $mgml^{-1}$ protein, 0.04 U) to give a final volume of 0.5 ml. The reaction was followed by reverse phase HPLC on a Techsphere 5C8 column (25 cm x 4.6 mm and a precolumn, 5 cm x 4.6 mm, elution with acetonitrile/10 mM ammonium acetate).

Synthesis of 1-β -D-2'-deoxyribofuranosyl-1,2,4-triazole-3-carboxamide, 2'-deoxyribavirin 65

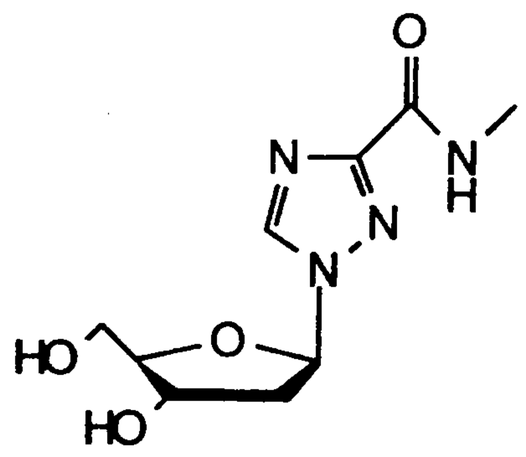


1,2,4-Triazole-3-carboxamide **18** (42 mg, 0.37 mmol) and thymidine (198 mg, 0.82 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (1.0 ml, 16.3 $mgml^{-1}$, 9 U) was added and the mixture incubated for 8 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 10 mM ammonium acetate). When the reaction had reached equilibrium (62% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel (methanol:dichloromethane, 10:90). Final purification was by preparative reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 20 mm, elution with 10 mM ammonium acetate, 8 $mlmin^{-1}$) to give 1-β-D-2'-deoxyribofuranosyl-1,2,4-triazole-3-carboxamide, 2'-deoxyribavirin **65** (29 mg, 35%) as a colourless solid, m.p. 111-112°C (lit.,[58] 112-113°C); $[\alpha]_D^{30}$ -0.6 (*c* 0.75 in MeOH); δ_H (400 MHz; MeOH-d^4) 2.50 (1 H, ddd, *J* 5.18, 6.68, 13.57, H-2'b), 2.80 (1 H, ddd, *J* 5.08, 6.25, 13.45, H-2'a), 3.69 (1 H, dd, *J* 5.38, 12.03, H-5'b), 3.79 (1 H, dd, *J* 4.10, 12.08, H-5'a), 4.05 (1 H, ddd appears as q, *J* 2 x 4.19, 5.35, H-4'), 4.59 (1 H, ddd appears as q, *J* 4.15, 2 x 5.15, H-3'), 6.34 (1 H, dd, *J* 5.00, 6.53, H-1') and 8.74 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 41.22 (C-2'), 63.23 (C-5'), 71.98 (C-3'), 89.70 (C-1'), 89.82 (C-4'), 146.11 (C-5), 158.08 (C-3) and 163.44 (C=O); *m/z* (CI) 229 ($(M+H)^+$, 7%), 211 (2), 130 (44), 113

((base+H)$^+$, 100), 98 (17) and 81 (19). The ^{1}H and ^{13}C NMR data compared favourably with that published by Sanghvi *et al.*[135]

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 8.74 ppm (H-5) caused enhancement due to H-1' (5.66%). Irradiation of the signal at 6.34 ppm (H-1') caused enhancement due to H-5 (4.35%) and H-4' (1.16%).

Synthesis of 1-β -D-2'-deoxyribofuranosyl-*N*-methyl-1,2,4-triazole-3-carboxamide 66*

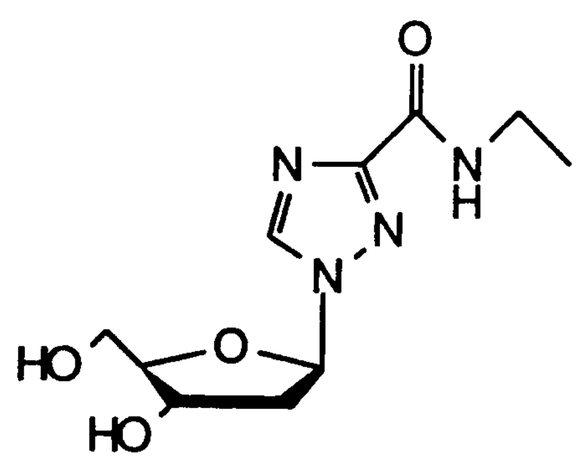


N-Methyl-1,2,4-triazole-3-carboxamide **19** (40 mg, 0.32 mmol) and 2'-deoxycytidine (154 mg, 0.63 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.5 ml, 16.3 mgml^{-1}, 9 U) was added and the mixture incubated for 7 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium (62% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel (methanol:dichloromethane, 5:95) to give 1-β-D-2'-deoxyribofuranosyl-*N*-methyl-1,2,4-triazole-3-carboxamide **66** (19 mg, 24%) as a colourless powder, Rf 0.38 (methanol:dichloromethane, 1:5); m.p. 173-179°C; $[\alpha]_D^{30}$ +10.35 (*c* 1.5 in MeOH); (Found: M$^+$ 243.1093. $C_9H_{14}N_4O_4+H^+$ requires 243.10945); δ_H (250 MHz; MeOH-d^4) 2.45 (1 H, ddd, *J*

5.20, 6.65, 13.55, H-2'b), 2.74 (1 H, ddd, *J* 5.10, 6.25, 13.58, H-2'a), 2.96 (3 H, s, Me), 3.64 (1 H, dd, *J* 5.38, 12.05, H-5'b), 3.75 (1 H, dd, *J* 4.08, 12.05, H-5'a), 4.00 (1 H, ddd appears as q, *J* 2 x 4.15, 5.28, H-4'), 4.55 (1 H, ddd appears as q, *J* 3 x 5.19, H-3'), 6.34 (1 H, dd, *J* 5.08, 6.58, H-1') and 8.74 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 26.33 (Me), 41.18 (C-2'), 63.21 (C-5'), 71.98 (C-3'), 89.65 (C-1'), 89.77 (C-4'), 146.10 (C-5), 158.20 (C-3) and 162.03 (C=O); *m/z* (CI) 243 ($(M+H)^+$, 15%), 193 (4), 144 (23), 127 (100) and 117 (8).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 6.29 ppm (H-1') caused enhancement due to H-5 (3.0%) and H-2'b (6.2%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-*N*-ethyl-1,2,4-triazole-3-carboxamide 67*

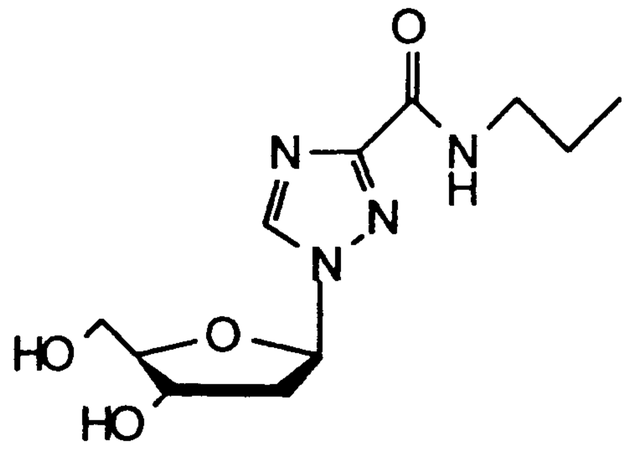


N-Ethyl-1,2,4-triazole-3-carboxamide **20** (20 mg, 0.14 mmol) and 2'-deoxycytidine (144 mg, 0.55 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.5 ml, 16.3 $mgml^{-1}$, 9 U) was added and the mixture incubated for 4 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 10 mM ammonium acetate). When the reaction had reached equilibrium (95% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel (methanol:dichloromethane, 2:98) to give 1-β-D-2'-deoxyribofuranosyl-*N*-ethyl-1,2,4-triazole-3-carboxamide **67** (33 mg, 89%)

as a colourless oil, $[\alpha]_D^{30}$ +8.30 (*c* 2.6 in MeOH); (Found: M^+ 257.125. $C_{10}H_{16}N_4O_4 + H^+$ requires 257.125); δ_H (400 MHz; MeOH-d^4) 1.26 (3 H, t, *J* 7.24, Me), 2.50 (1 H, ddd, *J* 5.16, 6.60, 13.56, H-2'b), 2.80 (1 H, ddd, *J* 2 x 6.16, 13.64, H-2'a), 3.46 (2 H, q, *J* 7.24, CH_2), 3.69 (1 H, dd, *J* 5.40, 12.04, H-5'b), 3.79 (1 H, dd, *J* 4.04, 12.00, H-5'a), 4.05 (1 H, ddd appears as q, *J* 4.20, 2 x 4.72, H-4'), 4.60 (1 H, ddd appears as q, *J* 4.95, 2 x 5.30, H-3'), 6.34 (1 H, dd, *J* 5.04, 6.56, H-1') and 8.73 (1 H, s, H-5); δ_C (400 MHz; MeOH-d^4) 14.9 (Me), 35.3 (CH_2), 41.2 (C-2'), 63.2 (C-5'), 72.0 (C-3'), 89.6 (C-1'), 89.75 (C-4'), 146.0 (C-5), 158.3 (C-3) and 161.2 (C=O); *m/z* (CI) 274 ($(M+NH_4)^+$, 8%), 257 ($(M+H)^+$, 27), 158 (100) and 141 (85).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 8.73 ppm (H-5) caused enhancement due to H-1' (2.9%). Irradiation of the signal at 6.34 ppm (H-1') caused enhancement due to H-5 (2.4%), H-4' (1.0%) and H-2'b (4.5%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-*N*-n-propyl-1,2,4-triazole-3-carboxamide 68*

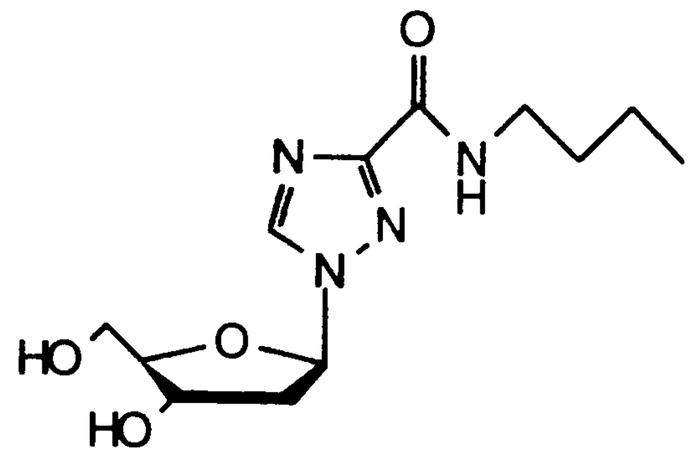


N-n-Propyl-1,2,4-triazole-3-carboxamide **21** (60 mg, 0.39 mmol) and 2'-deoxycytidine (191 mg, 0.78 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.75 ml, 16.3 $mgml^{-1}$, 9 U) was added and the mixture incubated for 3 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8

column, 25 cm x 4.6 mm, elution with 4% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium (54% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel with a gradient (methanol:dichloromethane, 10:90). Final purification was by preparative reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 20 mm, elution with 8% acetonitrile/10 mM ammonium acetate, 8 $mlmin^{-1}$) to give 1-β-D-2'-deoxyribofuranosyl-*N*-n-propyl-1,2,4-triazole-3-carboxamide **68** (30 mg, 29%) as a clear, colourless oil, $[\alpha]_D^{30}$ +3.07 (*c* 3.8 in MeOH); (Found: M^+ 271.1406. $C_{11}H_{18}N_4O_4+H^+$ requires 271.1408); λ_{max} (H_2O)/nm 209.6; δ_H (400 MHz; D_2O) 0.89 (3 H, t, *J* 7.44, Me), 1.58 (2 H, sextet, *J* 7.26, CH_2CH_3), 2.53 (1 H, ddd, *J* 5.24, 6.72, 14.00, H-2'b), 2.79 (1 H, ddd, *J* 5.36, 6.16, 14.08, H-2'a), 3.32 (2 H, t, *J* 7.04, $CH_2CH_2CH_3$), 3.66 (1 H, dd, *J* 6.16, 12.32, H-5'b), 3.76 (1 H, dd, *J* 3.88, 12.36, H-5'a), 4.09 (1 H, ddd appears as q, *J* 2 x 4.04, 6.08, H-4'), 4.60 (1 H, ddd appears as q, *J* 2 x 4.88, 5.72, H-3'), 6.35 (1 H, dd, *J* 5.24, 6.60, H-1') and 8.64 (1 H, s, H-5); δ_C (400 MHz; D_2O) 11.25 (Me), 22.55 (CH_2CH_3), 39.51 ($CH_2CH_2CH_3$), 41.93 (C-2'), 62.14 (C-5'), 71.27 (C-3'), 88.16 (C-1'), 88.63 (C-4'), 146.19 (C-5), 157.32 (C-3) and 174.16 (C=O); *m/z* (CI) 271 ($(M+H)^+$, 12%), 155 ($(base+H)^+$, 100), 117 (13), 99 (9) and 58 (11).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 8.65 ppm (H-5) caused enhancement due to H-1' (2.3%). Irradiation of the signal at 6.35 ppm (H-1') caused enhancement due to H-5 (4.59%), H-4' (1.15%) and H-2'b (7.89%). Irradiation of the signal at 3.75 ppm (H-5'a) caused enhancement due to H-3' (3.77%), H-4' (11.32%) and H-5'b (16.23%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-*N*-n-butyl-1,2,4-triazole-3-carboxamide 69*

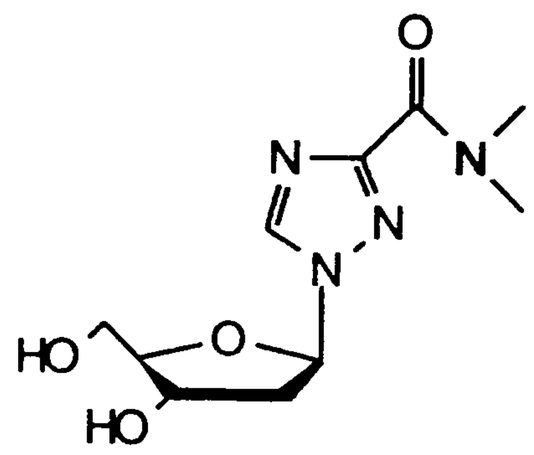


N-n-Butyl-1,2,4-triazole-3-carboxamide **22** (40 mg, 0.26 mmol) and 2'-deoxycytidine (184 mg, 0.78 mmol) were dissolved in citrate buffer (9 ml, 10 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (1 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.75 ml, 16.3 $mgml^{-1}$, 9 U) was added and the mixture incubated for 3 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 10% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium (73% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel (methanol:dichloromethane, 5:95) to give 1-β-D-2'-deoxyribofuranosyl-*N*-n-butyl-1,2,4-triazole-3-carboxamide **69** (46 mg, 62%) as a clear, colourless oil, $[\alpha]_D^{30}$ +9.64 (*c* 1.4 in MeOH); (Found: M^+ 285.1563. $C_{12}H_{20}N_4O_4+H^+$ requires 285.1564); δ_H (400 MHz; D_2O) 0.87 (3 H, t, *J* 7.40, Me), 1.31 (2 H, sextet, *J* 7.44, CH_2-γ), 1.54 (2 H, quintet, *J* 7.27, CH_2-β), 2.52 (1 H, ddd, *J* 5.12, 6.84, 14.00, H-2'b), 2.78 (1 H, ddd, *J* 5.24, 6.24, 14.08, H-2'a), 3.34 (2 H, t, *J* 7.02, CH_2-α), 3.65 (1 H, dd, *J* 6.08, 12.32, H-5'b), 3.75 (1 H, dd, *J* 3.92, 12.36, H-5'a), 4.08 (1 H, ddd appears as q, *J* 2 x 4.04, 6.16, H-4'), 4.44 (1 H, ddd appears as q, *J* 3 x 4.65, H-3'), 6.34 (1 H, dd, *J* 5.20, 6.68, H-1') and 8.63 (1-H, s, H-5); δ_C (400 MHz; D_2O) 13.68 (Me), 20.57 (CH_2-γ), 31.19 (CH_2-β), 39.56 (CH_2-α), 39.98 (C-2'), 62.17 (C-5'), 71.30 (C-3'), 88.20 (C-1'), 88.66 (C-4'), 146.18 (C-5), 157.33 (C-3) and 161.30 (C=O); *m/z* (CI) 285 ($(M+H)^+$, 11%), 186

(40), 169 ((base+H)$^+$, 100), 117 (5), 98 (21) and 72 (5).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 8.63 ppm (H-5) caused enhancement due to H-1' (6.00%) and H-2'a (0.91%). Irradiation of the signal at 6.34 ppm (H-1') caused enhancement due to H-5 (6.25%), H-4' (1.56%), CH_2-α (0.78%), H-2'a (0.40%) and H-2'b (7.42%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-*N*,*N*-dimethyl-1,2,4-triazole-3-carboxamide 70*

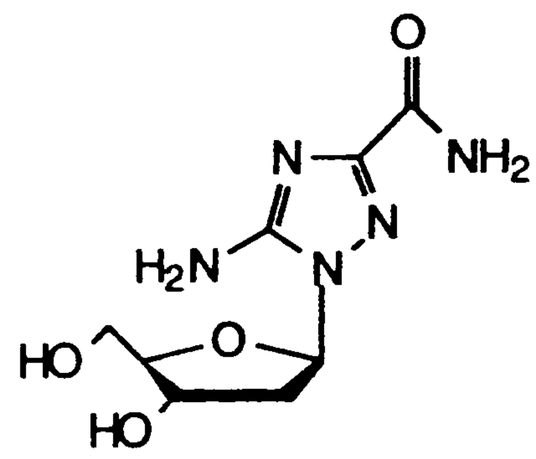


N,*N*-Dimethyl-1,2,4-triazole-3-carboxamide **25** (45 mg, 0.32 mmol) and 2'-deoxycytidine (210 mg, 0.86 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.75 ml, 16.3 mgml^{-1}, 9 U) was added and the mixture incubated for 11 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 4% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium (50% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel with a gradient (methanol:dichloromethane, 3:97 to methanol, 100). The last methanol fraction contained the product. Final purification was by preparative reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 20 mm, elution with 5% acetonitrile/10 mM ammonium acetate, 8 mlmin^{-1}) to give

1-β-D-2'-deoxyribofuranosyl-*N*,*N*-dimethyl-1,2,4-triazole-3-carboxamide **70** (36 mg, 43%) as a clear, colourless oil, $[\alpha]_D^{30}$ +25.0 (*c* 1.4 in MeOH); (Found: M^+ 257.1250. $C_{10}H_{16}N_4O_4+H^+$ requires 257.1251); λ_{max} (H_2O)/nm 201.6; δ_H (400 MHz; MeOH-d^4) 2.52 (1 H, ddd, *J* 5.28, 6.72, 13.64, H-2'b), 2.78 (1 H, ddd, *J* 4.84, 6.28, 13.64, H-2'a), 3.17 (3 H, s, Me, *syn*), 3.33 (3 H, s, Me, *anti*), 3.68 (1 H, dd, *J* 5.48, 11.96, H-5'b), 3.78 (1 H, dd, *J* 4.20, 11.96, H-5'a), 4.05 (1 H, ddd appears as q, *J* 2 x 4.24, 4.32, H-4'), 4.59 (1 H, ddd appears as q, *J* 2 x 5.60, 7.08, H-3'), 6.36 (1 H, dd, *J* 4.80, 6.64, H-1') and 8.77 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 36.25 (Me, *syn*), 39.26 (Me, *anti*), 41.15 (C-2'), 63.26 (C-5'), 72.64 (C-3'), 89.63 (C-1'/C-4'), 145.45 (C-5), 158.35 (C-3) and 163.38 (C=O); *m/z* (CI) 257 ($(M+H)^+$, 6%), 193 (3), 141 (100), 99 (12) and 81 (13).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 8.77 ppm (H-5) caused enhancement due to H-1' (4.40%). Irradiation of the signal at 6.35 ppm (H-1') caused enhancement due to H-5' (2.01%), H-4' (1.12%), H-2'a (0.45%) and H-2'b (6.27%). Irradiation of the signal at 3.67 ppm (H-5'b) caused enhancement due to H-3' (3.36%), H-4' (5.67%) and H-5'a (9.24%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-5-amino-1,2,4-triazole-3-carboxamide 71*

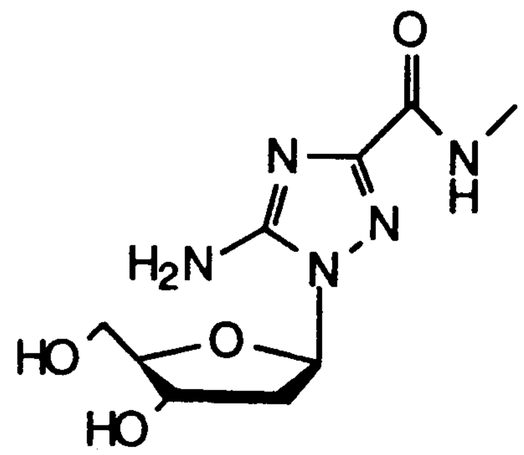


5-Amino-1,2,4-triazole-3-carboxamide **13** (18 mg, 0.14 mmol) and 2'-deoxycytidine (198 mg, 0.82 mmol) were dissolved in citrate buffer (4.5 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (0.5 ml,

10%). The crude *N*-deoxyribosyltransferase extract (0.5 ml, 16.3 $mgml^{-1}$, 9 U) was added and the mixture incubated for 9 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 10 mM ammonium acetate). When the reaction had reached equilibrium (75% conversion), the solution was lyophilised and the residue dissolved up in water and filtered under centrifugation to remove the protein. Purification was by preparative reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 20 mm, elution with 10 mM ammonium acetate, 8 $mlmin^{-1}$) to give 1-β-D-2'-deoxyribofuranosyl-5-amino-1,2,4-triazole-3-carboxamide **71** (22mg, 64%) as a colourless solid, m.p. 178°C (decomp); $[\alpha]_D^{30}$ +29.7 (*c* 8.0 in MeOH); (Found: M^+ 244.1046. $C_8H_{13}N_5O_4+H^+$ requires 244.10486); δ_H (400 MHz; MeOH-d^4) 2.32 (1 H, ddd, *J* 4.00, 6.52, 13.48, H-2'b), 2.83 (1 H, ddd appears as quintet, *J* 6.32, 6.52, 13.48, H-2'a), 3.67 (1 H, dd, *J* 5.00, 11.84, H-5'b), 3.75 (1 H, dd, *J* 3.96, 11.84, H-5'a), 4.00 (1 H, ddd appears as q, *J* 2 x 3.80, 4.76, H-4'), 4.58 (1 H, ddd appears as quintet, *J* 2 x 3.80, 6.24, H-3') and 6.14 (1 H, dd appears as t, *J* 6.36, H-1'); δ_C (250 MHz; MeOH-d^4) 39.59 (C-2'), 63.52 (C-5'), 72.60 (C-3'), 87.44 (C-1'), 89.17 (C-4'), 153.92 (C-5), 157.88 (C-3) and 164.37 (C=O); *m/z* (CI) 244 ($(M+H)^+$, 67%), 226 (3), 145 (51), 128 ($(base+H)^+$, 100), 112 (7), 98 (46) and 81 (48); *m/z* (EI) 177 (4%), 154 (11), 127 ($base^{+\cdot}$, 80), 97 (30) and 69 (100).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 6.14 ppm (H-1') caused enhancement due to H-4' (1.17%) and H-2'b (6.43%). Irradiation of the signal at 4.57 ppm (H-3') caused enhancement due to H-4' (1.64%), H-5'b (1.18%), H-5'a (1.77%), H-2'a (4.40%) and H-2'b (1.10%).

Synthesis of 1-β-D-2'-deoxyribofuranosyl-5-amino-*N*-methyl-1,2,4-triazole-3-carboxamide 72*

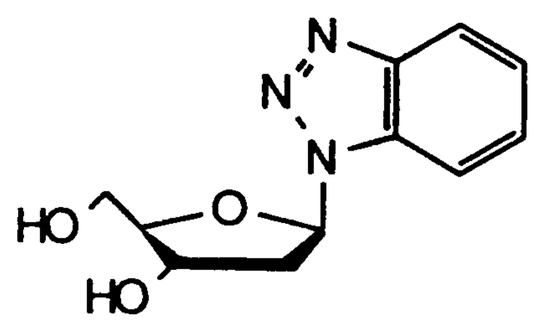


5-Amino-*N*-methyl-1,2,4-triazole-3-carboxamide **14** (43 mg, 0.31 mmol) and 2'-deoxycytidine (160 mg, 0.65 mmol) were dissolved in citrate buffer (22 ml, 20 mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.5 ml, 16.3 mg ml-1, 9 U) was added and the mixture incubated for 11 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium (66% conversion), the solution was lyophilised and the residue purified by flash chromatography on silica gel with a gradient (methanol:dichloromethane, 1:99 to 20:80) to give 1-β-D-2'-deoxyribofuranosyl-5-amino-*N*-methyl-1,2,4-triazole-3-carboxamide **72** (35 mg, 43%) as a colourless powder, Rf 0.22 (methanol:dichloromethane, 1:9); m.p. 125-127°C; $[\alpha]_D^{30}$ -8.5 (*c* 1.0 in MeOH); (Found: M^+ 258.1203. $C_9H_{15}N_5O_4+H^+$ requires 258.1204); δ_H (400 MHz; MeOH-d^4) 2.31 (1 H, ddd, *J* 4.04, 6.56, 13.48, H-2'b), 2.84 (1 H, ddd, *J* 6.32, 6.56, 13.48, H-2'a), 2.92 (3 H, s, Me), 3.66 (1 H, dd, *J* 5.08, 11.76, H-5'b), 3.75 (1 H, dd, *J* 4.00, 11.76, H-5'a), 4.00 (1 H, ddd appears as q, *J* 3 x 3.80, H-4'), 4.58 (1 H, ddd appears as quintet, *J* 2 x 3.80, 6.20, H-3') and 6.13 (1 H, dd appears as t, *J* 6.36, H-1'); δ_C (250 MHz; MeOH-d^4) 26.02 (Me), 39.59 (C-2'), 63.57 (C-5'), 72.60 (C-3'), 87.13 (C-1'), 89.27 (C-4'), 154.27 (C-5), 157.71 (C-3) and 162.63 (C=O); *m/z* (CI) 258 ($(M+H)^+$,

25%), 220 (5), 168 (8), 142 ((base+H)$^+$, 100), 117 (11), 98 (13) and 43 (27).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 6.13 ppm (H-1') caused enhancement due to H-4' (1.10%), H-2'a (1.10%) and H-2'b (6.50%). Irradiation of the signal at 4.00 ppm (H-4') caused enhancement due to H-1' (1.3%), H-3' (2.10%) and H-5' (6.1%).

Synthesis of 1-β-D-2'-deoxyribofuranosylbenzotriazole 73



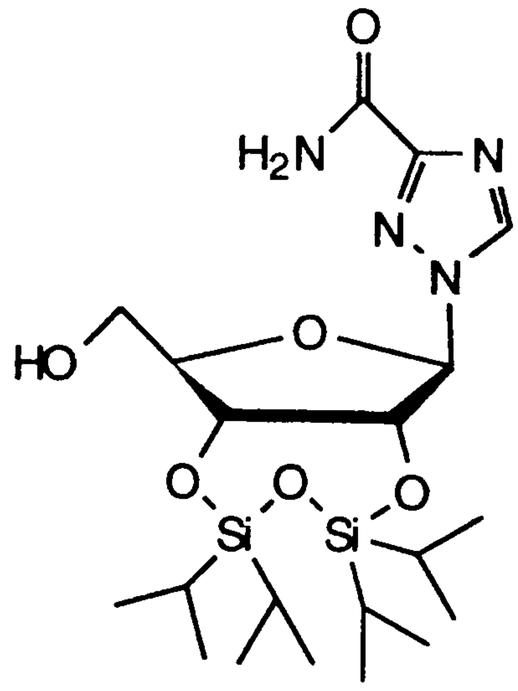
Benzotriazole **61** (43 mg, 0.36 mmol) and 2'-deoxycytidine (180 mg, 0.74 mmol) were dissolved in citrate buffer (45 ml, 0.2M, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (5 ml, 10%). The crude *N*-deoxyribosyltransferase extract (0.5 ml, 16.3 mgml^{-1}, 9 U) was added and the mixture incubated for 7 d at 40°C. The progress of the reaction was monitored by reverse phase HPLC analysis (Techsphere 5C8 column, 25 cm x 4.6 mm, elution with 2% - 10% acetonitrile/10 mM ammonium acetate). When the reaction had reached equilibrium, the solution was lyophilised and the residue purified by flash chromatography on silica gel (methanol:dichloromethane, 3:97) to give 1-β -D-2'-deoxyribofuranosylbenzotriazole **73** (42 mg, 50%) as a colourless solid, m.p. 149-150°C (lit.,[202] 150-151°C); λ_{max} (H_2O)/nm 211.7, 257.7 and 280.0 (lit.,[202] 257 and 281 nm); δ_H (400 MHz; D_2O) 2.61 (1 H, ddd, *J* 5.64, 6.40, 13.80, H-2'b), 3.20 (1 H, ddd, *J* 2 x 5.92, 13.92, H-2'a), 3.51 (1 H, dd, *J* 6.16, 12.32, H-5'b), 3.65 (1 H, dd, *J* 4.04, 12.28, H-5'a), 4.10 (1 H, ddd as appears q, *J* 2 x 4.32, 6.00, H-4'), 4.69 (1 H, ddd appears as q, *J* 2 x 5.08, 6.04, H-3'), 6.72 (1 H, dd appears as t, *J* 5.80, 6.24, H-1'), 7.42 (1 H, dd appears as t, *J* 7.56, 7.84, H-6),

7.55 (1 H, dd appears as t, *J* 7.40, 8.00, H-5), 7.74 (1 H, d, *J* 8.50, H-7) and 7.92 (1 H, d, *J* 8.52, H-4); δ_H (400 MHz; DMSO-d^6) 2.45 (1 H, ddd, *J* 4.40, 6.60, 13.34, H-2'b), 3.04 (1 H, ddd, *J* 6.16, 6.61, 13.35, H-2'a), 3.37 (1 H, dd, *J* 5.60, 11.56, H-5'b), 3.51 (1 H, dd, *J* 5.00, 11.56, H-5'a), 3.93 (1 H, ddd appears as br q, *J* 3 x 5.16, H-4'), 4.53 (1 H, ddd appears as m, *J* 2 x 4.12, 6.00, H-3'), 4.83 (1 H, br s, OH-2'), 5.45 (1 H, br s, OH-3'), 6.77 (1 H, dd appears as t, *J* 6.32, H-1'), 7.43 (1 H, dd appears as t, *J* 7.52, 7.76, H-6), 7.57 (1 H, dd appears as t, *J* 7.24, 7.96, H-5), 7.99 (1 H, d, *J* 8.36, H-7) and 8.07 (1 H, d, *J* 8.32, H-4); δ_C (400 MHz; DMSO-d^6) 40.59 (C-2'), 61.93 (C-5'), 70.79 (C-3'), 86.41 (C-1'), 88.30 (C-4'), 111.31 (C-7), 119.34 (C-4), 124.45 (C-5), 127.77 (C-6), 132.56 (C-8) and 145.5 (C-9); *m/z* (CI) 236 ($(M+H)^+$, 7%), 220 ($(base+H)^+$, 17), 172 (3), 155 (45), 120 (100), 98 (7) and 65 (12).

Nuclear Overhauser enhancement experiments (D_2O): Irradiation of the signal at 6.72 ppm (H-1') caused enhancement due to H-7 (4.7%) and H-2'b (6.5%). Irradiation of the signal at 4.10 ppm (H-4') caused no enhancement of any signals.

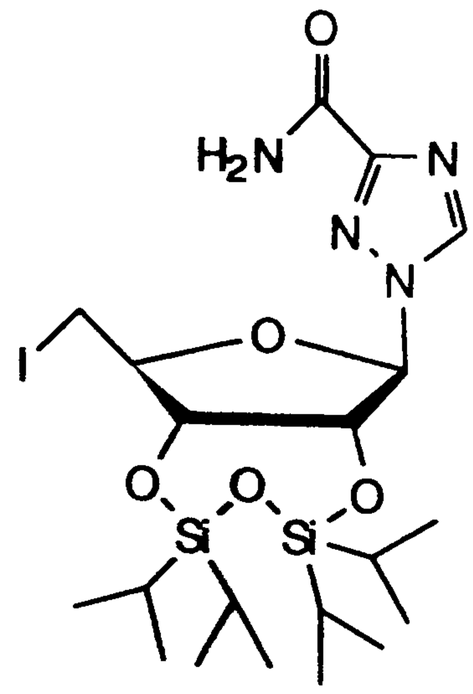
Nuclear Overhauser enhancement experiments (DMSO-d^6): Irradiation of the signal at 7.98 ppm (H-7) caused enhancement due to H-6 (9.23%), H-1' (5.06%) and H-2'a (1.79%). Irradiation of the signal at 8.06 (H-4) caused enhancement due to H-5 ppm (7.63%). Irradiation of the signal at 6.77 ppm (H-1') caused enhancement due to H-7 (5.31%), H-4' (1.36%) and H-2'b (7.48%).

Synthesis of 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin 168[226]*



To a suspension of ribavirin **7** (0.317 g, 1.3 mmol) and imidazole (0.219 g, 3.22 mmol) (previously freeze dried) in anhydrous DMF (10 ml) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.48 ml, 1.5 mmol). The clear mixture was stirred at room temperature under a nitrogen atmosphere for 12 h. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (methanol:dichloromethane, 3:97) to give 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **168** (0.705 g, 66%) as a colourless solid, m.p. 202-205°C (decomp); (Found: M^+ 487.2402. $C_{20}H_{38}N_4O_6Si_2+H^+$ requires 487.2409); δ_H (250 MHz; DMSO-d^6) 0.96-1.08 (28 H, m, Pr^i_2Si), 3.53 (1 H, dd, *J* 4.83, 11.90, H-5'a), 3.63 (1 H, dd, *J* 4.85, 11.90, H-5'b), 4.04 (1 H, ddd appears as q, *J* 3 x 4.47, H-4'), 4.61 (1 H, dd appears as t, *J* 2 x 4.46, H-3'), 4.85 (1 H, dd appears as t, *J* 2 x 4.83, H-2'), 5.07 (1 H, br t, *J* 5.97, OH-5'), 5.93 (1 H, d, *J* 4.83, H-1'), 7.68 (1 H, br s, NH-amide), 7.86 (1 H, br s, NH-amide) and 8.94 (1 H, s, H-5); δ_C (250 MHz; DMSO-d^6) 12.10-12.57 (*C*HSi), 16.84-17.29 (*C*H_3CHSi), 60.79 (C-5'), 72.32 (C-2'), 76.30 (C-3'), 86.03 (C-4'), 91.26 (C-1'), 145.47 (C-5), 157.56 (C-3) and 160.33 (C=O); *m/z* (EI FAB THIO) 487 ($(M+H)^{+\cdot}$, 15%), 391 (50), 357 (82) and 261 (100).

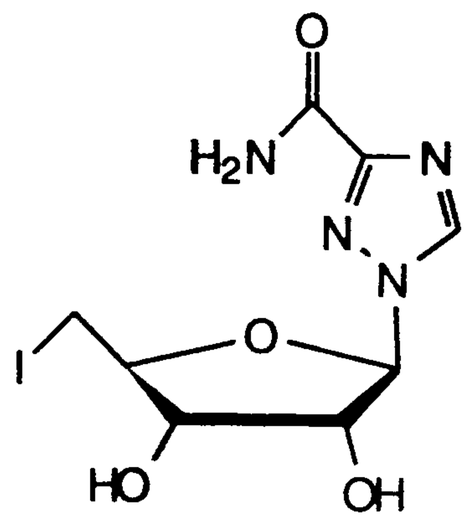
Synthesis of 5'-iodo-5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin 171[232]*



A solution of 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **168** (100 mg, 0.21 mmol) and freshly recrystallised[239] methyltriphenoxyphosphonium iodide **170** (186 mg, 0.41 mmol) in anhydrous DMF (8 ml) was stirred for 1 h at room temperature under a nitrogen atmosphere and covered to exclude light. The reaction was quenched with methanol (1 ml) and the solvent removed *in vacuo*. The residue was partitioned between ethyl acetate (40 ml) and sodium thiosulfate (2 x 7.5 ml, 10 M), washed with water (10 ml), brine (10 ml), dried over magnesium sulfate and the solvent removed *in vacuo* to give a clear colourless oil. Purification by flash chromatography on silica gel (methanol:dichloromethane, 2:98) and recrystallisation from methanol and dichloromethane gave 5'-iodo-5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **171** (77 mg, 62%) of colourless crystals, Rf 0.35 (methanol:dichloromethane, 1:10); m.p. 109-111°C; (Found: M^+ 597.143. $C_{20}H_{37}N_4O_5Si_2I+H^+$ requires 597.1424); δ_H (250 MHz; DMSO-d^6) 0.97-1.09 (28 H, m, Pr^i_2Si), 3.39 (1 H, dd, *J* 5.93, 10.47, H-5'a), 3.56 (1 H, dd, *J* 4.00, 10.48, H-5'b), 4.14 (1 H, ddd appears as q, *J* 2 x 3.97, 6.58, H-4'), 4.69 (1 H, dd appears as t, *J* 2 x 4.25, H-3'), 5.00 (1 H, dd appears as t, *J* 2 x 4.60, H-2'), 6.01 (1 H, d, *J* 4.70, H-1'), 7.70 (1 H, br s, NH-amide), 7.92 (1 H, br s, NH-amide) and 8.95 (1 H, s, H-5); δ_C (250 MHz; DMSO-d^6) 6.15 (C-5'),

12.17-12.51 (*C*HSi), 15.83-17.25 (*C*H_3CHSi), 75.52 (C-3'), 76.35 (C-2'), 84.85 (C-4'), 91.25 (C-1'), 146.01 (C-5), 157.74 (C-3) and 160.27 (C=O); *m/z* (CI) 597 ($(M+H)^+$, 42%), 553 ((M^+ - $CONH_2$), 100), 485 (30), 329 (52) and 113 ($(base+H)^+$, 23).

Synthesis of 5'-iodo-5'-deoxy-ribavirin 173[224]

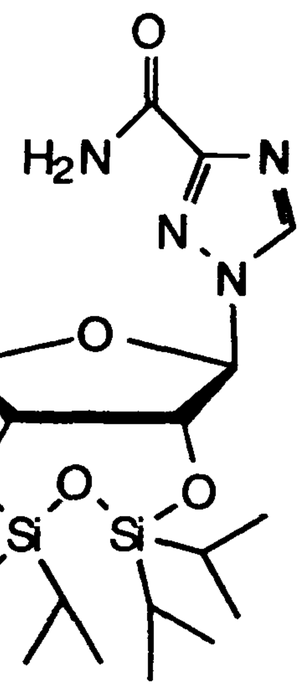


5'-Iodo-5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **171** (100 mg, 0.17 mmol) and ammonium fluoride (104 mg, 2.8 mmol) was refluxed in methanol (4 ml) at 60°C (bath temperature) for 40 min. Silica gel was added and the solution was evaporated to dryness. The residue was applied to a short column (3.0 cm long) and eluted with ethyl acetate (50 ml) followed by (methanol:ethyl acetate, 10:90). The product was recrystallised from methanol and dichloromethane to give 5'-iodo-5'-deoxy-ribavirin **173** (55.1 mg, 91.5%) as colourless crystals, Rf 0.14 (methanol:dichloromethane, 1:10); m.p. 180-184°C; (Found: C, 26.95; H, 3.02; N, 15.90. $C_8H_{11}N_4O_4I$ requires C, 27.13; H, 3.13; N, 15.82); δ_H (400 MHz; DMSO-d^6) 3.33 (1 H, dd, *J* 7.44, 10.54, H-5'b), 3.53 (1 H, dd, *J* 5.24, 10.56, H-5'a), 4.03 (1 H, ddd appears as br q, *J* 2 x 5.20, 7.44, H-4'), 4.19 (1 H, dd appears as br q, *J* 2 x 4.75, H-3'), 4.47 (1 H, dd appears as br q, *J* 2 x 4.32, H-2'), 5.72 (1 H, br d, *J* 5.20, OH-2'), 5.89 (1 H, d, *J* 3.72, H-1'), 6.01 (1 H, br d, *J* 5.36, OH-3'), 7.65 (1 H, br s, NH-amide), 7.88 (1 H, br s, NH-amide) and 8.87 (1 H, s, H-5); δ_C (400 MHz; DMSO-d^6) 7.56 (C-5'), 73.45 (C-3'), 74.49 (C-2'), 84.20 (C-4'), 91.29 (C-1'),

145.66 (C-5), 157.52 (C-3) and 160.34 (C=O); *m/z* (CI) 150 (11%), 130 (52), 113 ((base H)$^+$, 100), 99 (6) and 70 (13).

Nuclear Overhauser enhancement experiments: Irradiation of the signal at 5.88 ppm (H-1') caused enhancement due to H-5 (5.30%), H-2' (2.35%) and H-3' (1.47%). Irradiation of the signal at 4.48 ppm (H-2') caused enhancement due to H-5 (2.05%), H-1' (2.93%), OH-2' (1.76%), OH-3' (0.88%) and H-3' (6.45%). Irradiation of the signal at 4.19ppm (H-3') caused enhancement due to H-5 (0.70%), H-1' (0.30%), OH-2' (1.40%), OH-3' (1.74%), H-2' (6.60%), H-4' (2.1%) and H-5'a (1.4%).

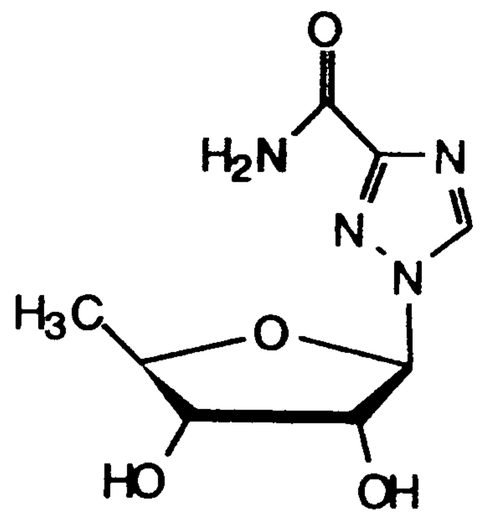
Synthesis of 5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin 172[148]*



A solution of 5'-iodo-5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **171** (90 mg, 0.15 mmol), 10% palladium on carbon (40 mg) and sodium acetate (33 mg, 0.40 mmol) in aqueous ethanol (20 ml, 50%) was hydrogenated at 15 psi and room temperature for 12 h. The catalyst was removed by filtering through a pad of celite and washed with methanol. The solvent was evaporated to give 5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **172** (45 mg, 63%) as a colourless powder, Rf 0.29 (methanol:dichloromethane, 1:19); (Found: M$^+$ 471.2459. $C_{20}H_{38}N_4O_5Si_2+H^+$ requires 471.2460); δ_H (250 MHz; $CDCl_3$) 1.02-1.05 (28 H,

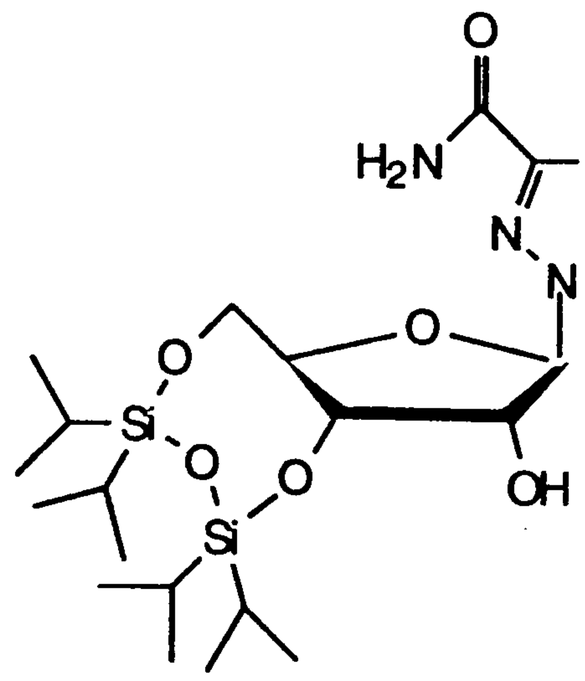
m, Pr^i_2Si), 1.41 (3 H, d, *J* 6.40, Me-5'), 4.13 (1 H, dd, *J* 4.38, 6.40, H-3'), 4.22 (1 H, quintet, *J* 6.40, H-4'), 4.78 (1 H, dd, *J* 3.05, 4.50, H-2'), 5.69 (1 H, br s, NH-amide), 5.81 (1 H, d, *J* 3.20, H-1'), 6.96 (1 H, br s, NH-amide) and 8.27 (1 H, s, H-5); δ_C (250 MHz; $CDCl_3$) 12.56-13.19 (*C*HSi), 16.83-17.32 (*C*H_3CHSi), 18.60 (C-5'), 77.10 (C-2'/3'), 80.88 (C-4'), 93.12 (C-1'), 143.90 (C-5), 157.30 (C-3) and 160.39 (C=O); *m/z* (CI) 471 ($(M+H)^+$, 100%), 453 (25), 427 (50), 354 (51), 329 (15) and 113 ($(base+H)^+$, 8).

Synthesis of 5'-deoxyribavirin 174[148]



A solution of 5'-iodo-5'-deoxyribavirin **173** (50 mg, 0.141 mmol) and 5% palladium on barium carbonate (32 mg) in aqueous ethanol (20 ml, 50%) was shaken in a Parr bombe hydrogenator at 18 psi for 16 h. The catalyst was removed by filtration through a pad of celite, washed with methanol and the filtrate was evaporated to dryness. Recrystallisation from methanol and dichloromethane gave 5'-deoxyribavirin **174** (27 mg, 84%) as colourless crystals, m.p. 131°C (lit.,[56] 132°C); δ_H (250 MHz; MeOH-d^4) 1.41 (3 H, d, *J* 6.025, Me-5'), 4.20 (2 H, m, H-3'/4'), 4.61 (1 H, dd, *J* 3.38, 4.30, H-2'), 5.96 (1 H, d, *J* 3.23, H-1') and 8.78 (1 H, s, H-5); δ_C (250 MHz; MeOH-d^4) 19.52 (C-5'), 76.32 (C-3'), 76.45 (C-2'), 82.45 (C-4'), 94.04 (C-1'), 147.16 (C-5), 158.50 (C-3) and 163.52 (C=O); *m/z* (CI) 246 ($(M+NH_4)^+$, 10%), 229 ($(M+H)^+$, 100), 145 (48), 130 (51) and 113 ($(base+H)^+$, 88); *m/z* (EI) 228 ($M^{+\cdot}$, 25%), 218 (28), 200 (58) and 169 (100).

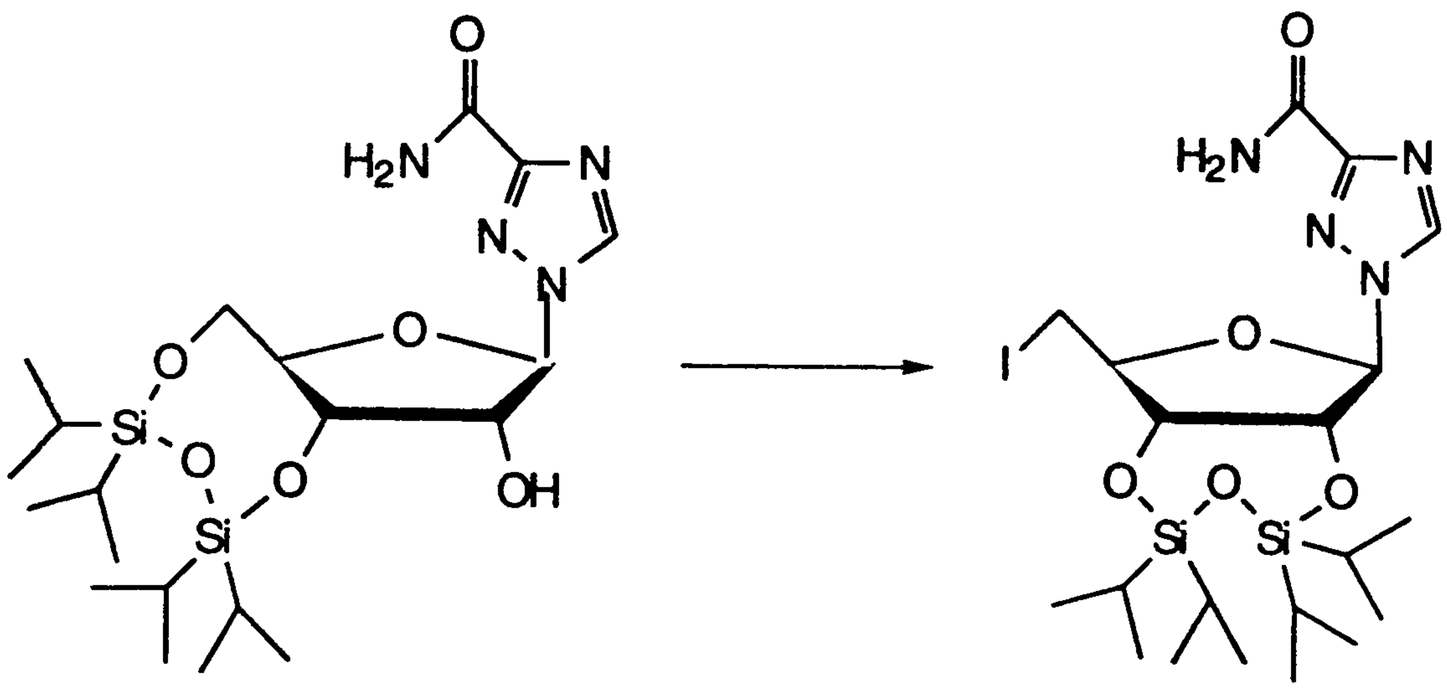
Synthesis of 3',5'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin 167[229]



Ribavirin **7** (1.5 g, 6.69 mmol) was made anhydrous by coevaporation with pyridine (10 ml). The residue was dissolved in anhydrous pyridine (45 ml) and cooled to 0°C with an ice/water bath. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (1.06 ml, 3.35 mmol) was added dropwise under a nitrogen atmosphere and stirred at room temperature for 24 h. The reaction was quenched with 5% ammonium carbonate (75 ml) under ice cooling and extracted with dichloromethane (2 x 75 ml), the extracts were washed with water (150 ml) and brine (25 ml), dried over magnesium sulfate and evaporated *in vacuo* to give a clear oil. Purification by flash chromatography on silica gel with a gradient (methanol:dichloromethane, 0.5:99.5 - 95:5) gave 3',5'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **167** (1.34g, 41%) as a colourless foam, m.p. 138°C, (lit.,[229] 136-138°C); δ_H (250 MHz; $CDCl_3$) 0.98-1.00 (28 H, m, Pr^i_2Si), 3.98 (1 H, dd, *J* 3.20, 15.20, H-5'a), 4.10 (1 H, dd, *J* 3.78, 15.98, H-5'b), 4.15 (1 H, ddd appears as q, *J* 3 x 3.48, H-4'), 4.45 (1 H, d, *J* 4.65, H-3'), 4.60 (1 H, dd, *J* 4.93, 7.85, H-2'), 5.92 (1 H, s, H-1'), 6.48 (1 H, br s, NH-amide), 7.01 (1 H, br s, NH-amide) and 8.40 (1 H, s, H-5); δ_C (250 MHz; $CDCl_3$) 12.562-13.22 (*C*HSi), 16.79-17.30 (*C*H_3CHSi), 61.36 (C-5'), 70.38 (C-2'), 75.53 (C-3'), 82.72 (C-4'), 92.15 (C-1'), 143.71 (C-5), 157.45 (C-3) and 160.86 (C=O); *m/z* (EI FAB

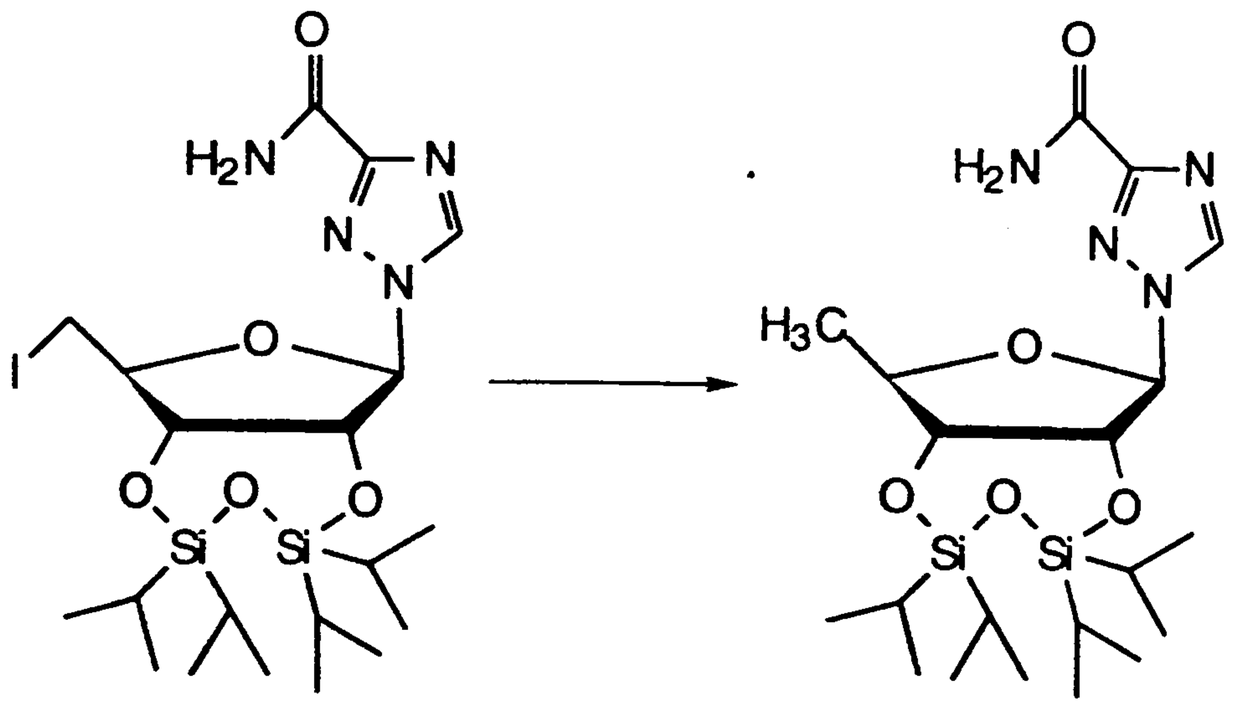
SODIUM THIOGLY) 509 ((M+H+Na)$^{+\cdot}$, 100%), 487 ((M+H)$^{+\cdot}$, 23) and 113 ((base+H)$^{+\cdot}$, 60). The ^{1}H and ^{13}C NMR data compared favourably with that published by Deyrup *et al.*[229]

Iodination of 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin[232*]



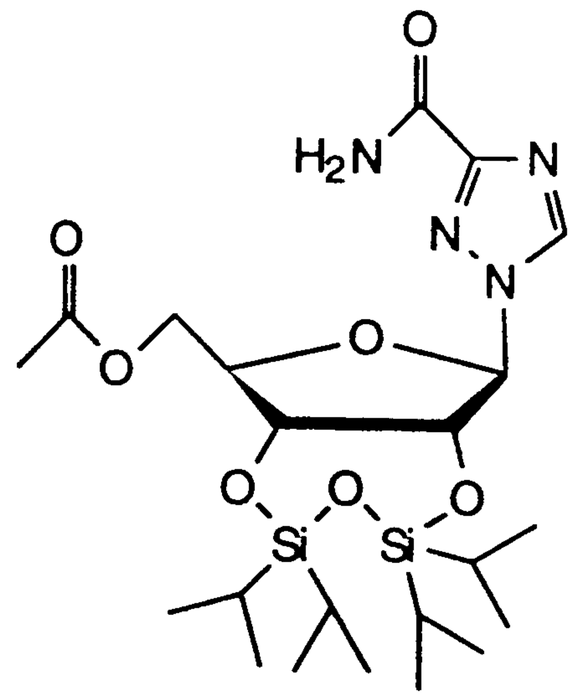
A solution of 3',5'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **167** (100 mg, 0.21 mmol) and freshly recrystallised[239] methyltriphenoxyphosphonium iodide **170** (186 mg, 0.41 mmol) in anhydrous DMF (8 ml) was stirred for 1 h at room temperature under a nitrogen atmosphere and covered to exclude light. The reaction was quenched with methanol (1 ml) and the solvent removed *in vacuo*. The residue was partitioned between ethyl acetate (40 ml) and sodium thiosulfate (2 x 7.5 ml, 10 M), washed with water (10 ml), brine (10 ml), dried over magnesium sulfate and the solvent removed *in vacuo* to give a clear oil. Purification by flash chromatography on silica gel (methanol:dichloromethane, 3:97) and recrystallisation from methanol and dichloromethane gave the iodinated 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin (74.5 mg, 61%) as colourless crystals, which was identical to that of 5'-iodo-5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **171** by NMR and TLC.

Hydrogenation of the iodination product of 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin[148]



A solution of 5'-iodo-5'deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **171** (74.5 mg, 0.125 mmol) and 5% palladium on barium carbonate (50 mg) and sodium acetate (15 mg) in aqueous ethanol (15 ml, 50%) was shaken in a Parr bombe hydrogenator at 18 psi for 5 h. The catalyst was removed by filtration through a pad of celite, washed with methanol and the filtrate was evaporated to dryness. Recrystallisation from methanol and dichloromethane gave the hydrogenated product (44.9 mg, 76.3%) as white crystals, which was identical to 5'-deoxy-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **172** by TLC and NMR.

Synthesis of 5'-*O*-acetyl-2',3'-*O*-1,1,3,3-tetraisopropyl-disiloxyl-ribavirin 169*



To a cooled (0°C) suspension of 2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **168** (60 mg, 0.123 mmol) in dry pyridine (5 ml) was added acetic anhydride (0.2 ml, 2.3 mmol). The reaction mixture was stirred at room temperature for 3 h. The pyridine was removed *in vacuo*, the residue was dissolved in ethanol and again evaporated to dryness. The oil was dissolved in dichloromethane (20 ml) and washed with aqueous saturated sodium hydrogen carbonate, water, dried over sodium sulfate and evaporated to dryness to give 5'-*O*-acetyl-2',3'-*O*-1,1,3,3-tetraisopropyldisiloxyl-ribavirin **169** (60.1 mg, 92.3%) as a colourless solid, m.p. 76-78°C; δ_H (250 MHz; DMSO-d^6) 0.96-1.05 (28 H, m, Pr^i_2-Si), 4.12 (1 H, dd, *J* 5.39, 11.34, H-5'b), 4.18 (1 H, ddd as m, H-4'), 4.34 (1 H, dd, *J* 3.16, 11.36, H-5'a), 4.74 (1 H, dd appears as t, *J* 4.85, H-3'), 4.85 (1 H, dd appears as t, *J* 4.10, H-2'), 6.04 (1 H, d, *J* 3.70, H-1'), 7.66 (1 H, br s, NH-amide), 7.85 (1 H, br s, NH-amide) and 8.98 (1 H, s, H-5); δ_C (250 MHz; DMSO-d^6) 12.08-12.47 (*C*H-Si), 16.76-17.13 (*C*H_3CH-Si), 20.46 (Me), 63.23 (C-5'), 72.98 (C-3'), 76.05 (C-2'), 82.30 (C-4'), 91.07 (C-1'), 145.88 (C-5), 157.77 (C-3), 160.20 (*C*ONH_2) and 170.08 (*C*OOMe); *m/z* (CI) 529 ($(M+H)^+$, 10%), 537 (12), 253 (58), 155 (100), 127 (75), 113 (13) and 85 (86).

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